CHEMISTRY AND BIOLOGICAL ACTIVITY OF NATURAL ANTIOXIDANTS

SUMMARY

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SUBMITTED FOR THE AWARD OF THE DEGREE OF

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BY

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ABSTRACT

The thesis entitled "Chemistry and Biological Activity of Natural Antioxidant" comprises of **seven** chapters dealing with the study of antioxidant activity of medicinally important plants. Anticancer activity of the leaf extracts of *Thuja orientalis* is also reported.

A broader definition of an antioxidant is "any substance which, when present at low concentrations compared to those of oxidizable substrates, significantly delays or prevents oxidation of those substrates." The term oxidizable substrates include DNA, lipids, proteins and carbohydrates which are essential building blocks of a biological system.

An increase in oxidative metabolism results in oxidative stress and produces a number of ROS. Antioxidants reduce oxidative stress and can play an important role conferring beneficial healthy effects.

Recently, there is an impetus for the search of powerful and non-toxic antioxidants from natural sources, especially crude drugs derived from medicinal plants. The plant phenolics have the ability to scavenge free radicals by single-electron transfer. Such **natural antioxidants** could prevent the formation of ROS related disorders in human being and help in

avoiding the use of synthetic compounds, which may be carcinogenic and harmful to the lungs and liver.

Chapter 1 includes a review of literature on the chemistry, biology and mechanism of action of antioxidants. In the end of this chapter the objective of the present study is presented.

In **Chapter-2**, the methods used for the evaluation of antioxidant activity and estimation of total phenolic contents in plant extracts containing natural antioxidants are described. The methodology used for the study of anticancer activity of *Thuja orientalis* against cervical carcinoma is also described.

The total phenolic content and antioxidant activity of *Thuja orientalis* is discussed in **Chapter-3**. The aqueous extract of *Thuja orientalis* shows higher total phenolic content and antioxidant activity as compared to the ethanol extract. Water extract of *T. orientalis* at a concentration of 500 μg/ml was able to scavenge 68.59% of DPPH free radical while alcohol extract at the similar concentration was able to scavenge 59.73% of DPPH free radical. Thus, plant phenolics are extracted in better amount by using water as a solvent in the case of *Thuja orientalis*.

The evaluation of anticancer activity of the leaf extracts of *Thuja orientalis* is discussed in **Chapter-4**. The results showed that the ethanol

extract of the leaves of *Thuja orientalis* (TO-al) induced apoptosis and inhibited the proliferation of cancer cells.

The antioxidant activity and total phenolic content of different solvent extracts of the edible fruits of *Ficus racemosa* is discussed in **Chapter-5**. Among the five solvent extracts tested i.e., methanol, ethyl acetate, acetone, chloroform and petroleum ether, methanol extract (F-Me) showed highest antioxidant activity and total phenolic content. The decreasing order of antioxidant activity is as follows: Methanol extract (F-Me)>Ethyl acetate extract (F-EA)>Acetone extract (F-Ac)>Chloroform extract (F-Chl)>Petroleum ether extract (F-Pet). At a concentration of 150 μg/ml, methanol extract is able to scavenge 52.16% of DPPH free radical, while at the same concentration ethyl acetate, acetone, chloroform and petrol extract showed 23.68%, 19.24%, 15.30% and 14.26% radical scavenging activity, respectively. Thus, selective extraction of antioxidant from natural sources by appropriate solvent is very important in obtaining fractions with high antioxidant activity.

In **Chapter 6**, the antioxidant activity and total phenolic content of some medicinal herbs from the family Euphorbiaceae, Asteraceae, Scrophuliaraceae and Solanaceae is discussed. The antioxidant activity of the medicinal herbs is not dependent on the families to which these herbs are

belonging, the berries of *Solanum nigrum* (Family Solanaceae) are showing highest antioxidant activity and leaf extract of *Pluchea lanceolata* is showing second highest activity.

In **Chapter 7**, the antioxidant activity and total phenolic content of eleven edible flowers is discussed. Among the eleven edible flowers tested, ethanol extract of *Tropaeolum majus* is showing highest antioxidant activity and total phenolic content followed by *Rosa indica, Viola wittrokiana, Hibiscus rosasinensis, Dianthus caryophyllus, Allium tuberosum*, *Dianthus barbatus, Raphanus sativus, Coriandrum sativum* and *Brassica campestris.*

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Dedicated

To

My Parents

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CERTIFICATE

This is to certify that the work discussed in the thesis entitled "Chemistry and Biological Activity of Natural Antioxidants" is the original work carried out by Ms.Iram Nizam under my supervision. It is suitable for submission for the award of Ph.D. degree in Chemistry.

(Prof.M.Mushfiq)

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Iram Nizam
(Iram Nizam)

ABBREVIATION

AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
BHT	Butylated hydroxytoluene
CAT	Catalase
CHD	Coronary heart diseases
DEVD-fmk	Aspartatic acid-Glutamic acid-Valine-Aspartic acid- fluoromethyl ketone
DEVD-pNA	Aspartatic acid-Glutamic acid-Valine-Aspartic acid -para-nitroanilide
DNA	Deoxyribonucleic acid
DPPH	1, 1-Diphenyl-2-picrylhydrazyl radical
DPX	Distrene dibutylphthalate xylol
ECL	Enhanced Chemiluminescense
EDTA	Ethylene Diamine Tetraacetic Acid
EGCG	Epigallocatechin Gallate
GAE	Gallic Acid Equivalents
GPx	Glutathione peroxidases
GSH	Glutathione
HDL	High Density Lipoprotein
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
HPLC	High Performance Liquid Chromatography
IETD-fmk	Isoleucine- Glutamic acid-Threonine-Aspartic acid- fluoromethyl ketone
IETD-pNA	Isoleucine- Glutamic acid-Threonine-Aspartic acid- para-nitroanilide
LDL	Low Density Lipoprotein
LEHD-fmk	Leucine-Glutamic acid-Histidine-Aspartic acid- fluoromethyl ketone

LEHD-pNA	Leucine-Glutamic acid-Histidine-Aspartic acid-para-nitoanilide
MDA	Malondialdehyde
ml	Millilitre
ml	Millilitre
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium
ORAC	Oxygen Radical Absorbance Capacity
PARP	Poly (ADP-ribose) polymerase
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate buffered saline
PUFA	Polyunsaturated Fatty Acid
RBC	Red Blood Corpuscles
ROS	Reactive Oxygen Species
RPMI-60	Roswell Park Memorial Institute-60
S.D.	Standard Deviation
S.E.M.	Standard Error of Mean
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TBST	Tris-Buffered Saline Tween-20
TRAP	Total radical trapping antioxidant parameter
UV	Ultra Violet
XO	Xanthine Oxidase
µg	Microgram
µl	Microlitre
µM	Micromolar

CONTENTS

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CHAPTER -1

ANTIOXIDANTS: A PREVIEW

1.1. Introduction

The oxygen we breathe is needed by our cells to generate energy. In fact, the very word "oxygen" has come to mean something that is vital for existence. This life-giving gas is the terminal acceptor of electrons during respiration, which is the main source of energy in aerobes. Although most of the oxygen used by our bodies to create energy is incorporated into harmless water molecules, up to 2 percent may be spun off into our cells as highly charged molecules known as free radicals or **reactive oxygen species** (ROS), which include hydroxyl radical ($\dot{O}H$), superoxide ($O_2^{\bullet-}$) and peroxyl ($ROO^{\bullet}$) radical. ROS, formed during metabolism or through the action of ionizing radiation, can interact with biomolecules and ultimately lead to an onset of degenerative diseases such as cancers, cardiovascular diseases and neurodegenerative disorders. To protect against the destructive action of free radicals, nature has created an **antioxidant defense system** composed of a group of compounds and enzymes potent enough to remove free radicals before they could cause tissue damage. Some antioxidants are produced in the body, while others must be sequestered from the diet or through supplementation (Ames et al., 1993).

Recently, there is an impetus for search of powerful and non-toxic antioxidants from natural sources, especially crude drugs derived from

medicinal plants. The plant phenolics have the ability to scavenge free radicals by single-electron transfer (Hirano et al, 2001). Such **natural antioxidants** could prevent the formation of ROS related disorders in human being and help in avoiding the use of synthetic compounds, which may be carcinogenic and harmful to the lungs and liver (Branen, 1975).

1. 2. Types and Sources of ROS

A number of ROS are continuously generated in living system as a consequence of normal metabolic processes. These can be produced in two ways: by **enzymatic reactions** involving xanthine oxidase (XO), NADPH oxidases and lipoxygenases: by **non-enzymatic** sequence of reactions such as the catalytic action of free transition metals (for example iron and copper), by the toxic action of certain chemicals such as doxorubicin, by the attack of electrons leaked from the mitochondrial electron transport chain and by the effect of radiation including UV light and radon (Ra) gas. Different types of ROS generated include both free radical and non-radical species (Bakonyi and Radak, 2004; Kerr et al., 1996; Ahmad, 1996; Mc Cord, 1993; Kehrer, 1993). The major ROS have been listed in Table 1.1

Table 1.1: Types of Reactive Oxygen Species (ROS)

Types of ROS	Symbol
Superoxide	$O_2^{\bullet-}$
Hydroxyl	$OH^{\bullet}$
Alkoxyl	$LO^{\bullet}/RO^{\bullet}$
Peroxyl	$LOO^{\bullet}$ / $ROO^{\bullet}$
Nitric oxide	$NO^{\bullet}$
Thiyl radical	$RS^{\bullet}$
Hydrogen peroxide	H_2O_2
Hypochlorous acid	HOCl
Ozone	O_3
Singlet oxygen	$^1O_2$
Peroxynitrite	$ONOO^{\bullet}$
Lipid peroxide	LOOH

Alkyl, alkonyl, alkylperoxyl radicals and lipid peroxide ($R^{\bullet}$, $RO^{\bullet}$, $ROO^{\bullet}$, and LOOH) are the products of attack of free radicals on lipid and fatty acid molecules. These biomolecules are more susceptible towards free radical attack due to the presence of unsaturation.

Superoxide anions ($O_2^{\bullet-}$) are the reduced form of oxygen. Though $O_2^{\bullet-}$ itself is not damaging, it plays an important role in the formation of ROS like $OH^{\bullet}$, H_2O_2 and nitric oxide radicals, and also acts as a reductant

for transition metals (Cheeseman and Slater, 1993; Fridovich, 1997). Superoxide radical anion can decrease the activity of certain enzymes including some antioxidant defense enzymes such as catalase, glutathione peroxidase, and several in the energy metabolism scheme such as NADH dehydrogenase (Willcox et al., 2004).

There are three main sites of $O_2^{\bullet-}$ production in cells: the mitochondria, endoplasmic reticulum, and cell cytoplasmic membranes. In mitochondria, besides other enzymes of the electron transfer chain, two enzymatic sites have been clearly identified as major sources for one electron reduction to oxygen: ubiquinone-cytochrome C reductase, which involves autooxidation of the ubisemiquinone (Sun and Trumpower, 2003; Han et al., 2001; Nohi and Jordan, 1998) and NADH dehydrogenase, which involves autooxidation of semi-flavin cofactor (Kalinowki and Malinski, 2004; Aust et al., 1972).

In the membrane of the endoplasmic reticulum, $O_2^{\bullet-}$ is produced by the oxycomplex of cytochrome P-450 and the action of NADH- cytochrome, P-450 reductase (Henderson and Chappell, 1996). In plasma, NADPH oxidase is involved in the generation of $O_2^{\bullet-}$ radicals by transferring one electron to molecular oxygen by the enzyme electron transfer chain reaction (Pithon-Curi et al., 2002). Xanthine oxidase has also been proposed to be an

important source of $O_2^{\bullet-}$ generation in reperfused tissues. It uses molecular oxygen as its acceptor, producing superoxide anion (Prigmore et al., 1995, Mc Cord and Omar, 1993; Viel et al., 2008).

Hydroxyl radical ($OH^{\bullet}$) is an extremely reactive radical species that can react with every bio-molecule. A number of sources generate these radicals. Hydrogen peroxide, $O_2^{\bullet-}$ and transition metals (Fe^{+2} and Cu^{2+}) are generally involved in the generation of $OH^{\bullet}$. Hypochlorous acid is another source of hydroxyl radicals (Mc Cormick et al., 1998; Cheeseman and Slater, 1993).

Hydrogen peroxide (H_2O_2) is an oxidizing agent and a main source of hydroxyl radicals as mentioned earlier. It produces $OH^{\bullet}$ through Haber-Weiss and Fenton reactions in the presence of $O_2^{\bullet-}$ and Fe^{+2}, respectively.

$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^-$ (Haber-Weiss reaction)

$H_2O_2 + Fe^{+2} \rightarrow OH^{\bullet} + OH^- + Fe^{+3}$ (Fenton reaction)

H_2O_2 also produces another oxidizing agent, hypochlorous acid (Halliwell and Gutteridge, 1992). Hypochlorous acid (HOCl) is a potent antibactericidal agent produced by the activated polymorphonuclear cells. It is generated by the action of myeloperoxidase on chloride ions in the presence of H_2O_2:

$$H_2O_2 + Cl^- \xrightarrow{\text{myeloperoxidase}} HOCl + OH^\bullet$$

It can cross cell membrane and, in the presence of transitional metal ions, it can generate hydroxyl radicals ($OH^\bullet$) by reacting with superoxide anion radicals and ferrous ion (Pullar et al., 2001; Arouma, 1994):

$$HOCl + Fe^{+2} \rightarrow OH^\bullet + Cl^\bullet + Fe^{+3}$$

$$HOCl + O_2^{\bullet} \rightarrow OH^\bullet + Cl + O_2$$

Nitric oxide ($NO^\bullet$) is a common gaseous free radical. It plays an important role in vascular physiology and is also known as endothelium derived relaxing factor. Vascular endothelium produces nitric oxide, like neutrophils and macrophages, from arginine, using the enzyme nitric oxide synthetase (Mazzetti et al., 2001; Moncado et al., 1991).

Peroxynitrite ($ONOO^\bullet$) is produced due to the release of significant quantity of $NO^\bullet$ and $O_2^{\bullet -}$ from activated macrophages and neutrophils during the inflammatory response (Grace et al., 1998).

$$O_2^{\bullet -} + NO^\bullet \rightarrow ONOO^\bullet$$

Singlet oxygen ($^1O_2$) is an electronically excited and mutagenic form of oxygen. It is generated during exercise, by radiations, by the action of peroxidases or lipoxygenases, and by the reaction of hydrogen peroxide with

hypochlorite or peroxynitrite, as well as during the respiratory burst of phagocytes (Mascio et al., 1994; Steinbeck et al., 1993).

In mammalian cells, singlet oxygen can be generated during oxidative stress and it is able to attack many cellular molecules such as amino acids, nucleic acid bases and membrane lipids. Sunlight contains high energy short-wavelength ultraviolet photons (comprising the UVB spectra, 290-320 nm) which are potentially detrimental because of their destructive interactions with many cellular biomolecules.

Chronic exposure to sunlight is thus a significant causative factor in the development of skin cancer (Estevam et al., 2004).

1. 3. Adverse Effects of Free Radicals

Because of the immense reactivity of free radicals, they can easily react with several biomolecules including DNA, lipids, proteins and carbohydrates. ROS react with the biomolecules, leading to local injury and eventual organ dysfunction. They also accelerate the aging and related degenerative processes. Moreover, ROS are also involved in the promotion of heart diseases, chronic inflammation, and cancers (Ivanova and Ivanov, 2000).

The susceptibility of biological membranes to peroxidation is due to the presence of polyunsaturated fatty acids (PUFA). The presence of double

bonds in PUFA weakens the C-H bond of the adjacent carbon atom (allylic carbons) and facilitates the hydrogen abstraction step, which initiates the peroxidation reactions (Aitken and Fischer, 1994). Cell membranes contain a variety of PUFA such as linoleic, linolenic and arachidonic acids, mainly in the form of esters with phospholipids, triglycerides, or with cholesterol. Overall attack of one reactive free radical on PUFA molecule can convert multiple fatty acid side chains into lipid peroxides, damage membrane proteins, make the membrane leaky and eventually cause breakdown of the membrane (Schafer et al., 2000; Cheeseman and Slater, 1993).

Protein and nucleic acids are generally less susceptible to free radical attack than PUFAs and have less possibility to take part in the progression of chain reactions. This can happen only if radicals are allowed to accumulate, or if the damage is focused on a particular site of the protein (Leeuwenburgh et al., 1998; Stadtman and Oliver, 1991).

Deoxyribonucleic acid (DNA) is a sensitive target for free radical-mediated damage in a living system. A free radical can damage the specific site of DNA, leading to breaking of strands, or it might delay the repair before replication occurs, leading to mutations (Cheeseman and Slater, 1993; Halliwell, 1999). It has been reported that free radicals may be involved in cell death or sub-lethal injuries such as mutations, chromosomal aberrations

or carcinogenesis by damaging DNA and the DNA repair processes (Aust and Eveleigh, 1999; Van Rensburg et al., 1992).

1. 4. Mechanism of Action of Free Radicals

There are three main stages in free radical mediated reactions: initiation, propagation and termination. Initiation starts with the abstraction of a hydrogen atom from the bio-molecule. For example fatty acid (LH) can be converted into radicals ($L^{\bullet}$). The hydroxyl ($OH^{\bullet}$), alkylperoxyl ($ROO^{\bullet}$), and alkoxyl ($RO^{\bullet}$) radicals are all capable of oxidizing PUFAs. Extremely rapid addition of oxygen to the fatty acid radicals then generate peroxyl radicals ($LOO^{\bullet}$) that propagate the reaction by initiating a new chain of oxidation with the formation of lipid hydroperoxide (LOOH). This chain reaction continues till an antioxidant interrupts it through scavenging the radicals: the termination step (Schafer et al., 2000; Aitken and Fischer, 1994).

1. 5. Antioxidants

To protect cells and organs from the oxidative stress induced by ROS, living organisms have evolved with an extremely efficient and highly sophisticated protective system, the so-called **"antioxidant defense system"**. It involves a variety of components, both involving components of

endogenous and exogenous in origin. These components function interactively and synergistically to neutralize free radicals (Percival, 1998). A broader definition of an antioxidant is **"any substance which, when present at low concentrations compared to those of oxidizable substrates, significantly delays or prevents oxidation of those substrates."** The term oxidizable substrates include DNA, lipids, proteins and carbohydrates which are essential building blocks of a biological system (Halliwell et al., 1995).

An increase in oxidative metabolism results in oxidative stress and produces a number of ROS. Antioxidants reduce oxidative stress and can play an important role conferring beneficial healthy effects (Vaya and Aviram, 2001). High dietary intake of proven antioxidants can significantly lower the risk of several chronic diseases such as heart diseases, cancers and cataracts.

1.5.1. Classification of Antioxidants

A variety of antioxidants are collectively required for the removal of free radicals to protect the body from adverse effects of ROS. Certain enzymes as well as non-enzymatic cellular molecules are involved in the detoxification of ROS. Based on the nature of antioxidants, the human

antioxidant system can be categorized into two broader classes: enzymatic and non-enzymatic (Vaya and Aviram, 2001).

A - Enzymatic Antioxidants

The major primary intracellular endogenous antioxidant defenses are the enzyme system. This antioxidant enzymatic system includes superoxide dismutases (SODs), catalase (CAT), and glutathione peroxidase (GSH px) (Yang et al., 1999; Halliwell and Gutteridge, 1990).

Superoxide dismutases (SODs) have been found in three isoforms. Manganese containing SOD (MnSOD) is a tetrameric protein that is localized in the mitochondrial matrix. It plays a key role in scavenging $O_2^{\bullet-}$ generated from the electron transport chain. Copper and zinc containing SOD (CuZnSOD) is a dimer protein that is localized in the cell cytoplasm. It is thought to remove $O_2^{\bullet-}$ generated by endoplasmic reticulum and cytosolic oxidases. The extracellular SOD is a tetrameric protein found in the extracellular space (Noor et al., 2002). SODs catalyze the dismutation of superoxide into H_2O_2 (Ivanova and Ivanov, 2000; Waris and Ahsan, 2006).

$$2O_2^{\bullet-} + 2H^{\bullet} \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

Catalase (CAT) is located in peroxisomes and mitochondria. It is a large tetrameric protein which removes H_2O_2 by catalyzing its conversion into water (Krinsky, 1992).

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Glutathione peroxidases (GPx) are a group of selenium- dependent enzymes. Four types of GPx cellular GPx (cGPx), gastrointestinal GPx (GIGPx), extracellular GPx

(eGPx), and phospholipid hydroperoxide GPx (PHGPx)) have been described. All GPx require glutathione as a cofactor and secondary enzymes, such as glutathione reductase and glucose-6-phosphate dehydrogenase, to function. Glucose-6-phosphate dehydrogenase generates NADPH to recycle the GSH (Takebe et al., 2002; Ivanova and Ivanov, 2000).

$$2GSH + H_2O_2 \xrightarrow{GP_x} GSSG + 2H_2O$$

B- Non-Enzymatic Antioxidants

Non-enzymatic antioxidants may be further classified into two groups: endogenous and exogenous antioxidants. The major extracellular endogenous antioxidants found in human plasma are transition metal binding proteins. These include ceruloplasmin, transferrin, hepatoglobin and albumin. They bind with transition metals and hence control the production

of metal-catalyzed free radicals. Albumin and ceruloplasmin are the copper ions sequesters. Hepatoglobin binds with hemoglobin while ferritin and transferrin bind with free iron (Ivanova and Ivanov, 2000; Halliwell and Gutteridge, 1990). Lipoic and uric acids, bilirubin, ubiquinone and glutathione are non-protein endogenous antioxidants which inhibit the oxidation processes by scavenging free radicals (Shahidi, 1997).

Many effective exogenous antioxidants are generally of dietary origin. The best known are vitamins such as ascorbic acid, vitamin E, carotenoids, and polyphenols. These molecules can inhibit oxidative reactions by scavenging free radicals, while certain compounds may chelate redox active metals or inhibit particular oxidative enzymes. Vitamin E is a lipid soluble, chain breaking antioxidant which reacts with lipid peroxyl radicals to yield a relatively stable lipid hydroperoxide and thus protects against membrane lipid peroxidation (Magiure et al., 1989). On the other hand, vitamin C has multiple antioxidant properties, including the ability to regenerate α-tocopheryl radicals at the membrane surfaces. It also scavenges other free radicals and certain non-radicals such as HOCl (Packer and Cadenas, 2002).

1.6. Mechanism of Action of Antioxidants

Antioxidants can remove both free radical and non-radical species through different modes of action depending upon which ROS is required

for neutralization. On the basis of the nature of ROS, two basic mechanisms have been proposed for the action of antioxidants: mechanism of removal of ROS initiators and a chain breaking mechanism.

A- Mechanism of Removal of ROS initiators

This process is mainly based on the inhibition of the enzymes involved in the production of ROS. Xanthine oxidase (XO) is one of the major sources of superoxide anions production (Borges and Roleira, 2002). Lipoxygenase, during arachidonic metabolic pathway, produces lipid peroxides (Dailey and Imming, 1999). Antioxidants inhibit these enzymes so that they are not available to harm the cellular system. The other main sources of free radicals are free transition metal ions which, by virtue of their attachment with carrier proteins, act as pro-oxidants and are therefore toxic to the body. There are several metal binding proteins that chelate transition metals, which are capable of reacting with hydroperoxides to produce free radicals:

$$HOOH/LOOH + Fe^{2+} \longrightarrow LO^{\bullet}/OH^{\bullet} + OH^{-} + Fe^{+3}$$

The proteins which bind with the transition metal ion include transferrin, lactoferrin and ferritin. These proteins function to keep iron-induced oxidant stress in control. Ceruloplasmin and albumin proteins are the copper and iron sequesters, respectively (Vaya and Aviram, 2001; Krinsky, 1992).

B- Chain Breaking Mechanism of Action

Antioxidants scavenge free radicals by donating an electron to them while being oxidized themselves during the process. This is known as "chain breaking antioxidation" (CBA). The chain reactions of free radical can be stopped with the help of antioxidants, which can neutralize free radicals formed during the overall reaction. Most of the polyphenolics work through the chain breaking (CB) mechanism of action. Vitamin C and E, carotenes, flavonoids and coumarins are examples of chain breaking antioxidants (CBA).

Vitamin E (tocopherol, TH_2) is the most widely distributed lipid soluble antioxidant in nature. Its main function is to prevent the peroxidation of membrane phospholipids and to avoid cell membrane damage (Maguire et al., 1989). A detailed study of the effects of benzene ring subsituents on the rate of radical scavenging reaction showed that the reaction is accelerated by the presence of a 4-methoxy group and C-2 and C-6 methyl groups. The presence of C-1 hydroxyl group contributes to donating $H^{\bullet}$, whereas functional groups at C-4, C-2, and C-6 stabilize the resulting tocopheryl radical (Shahidi, 1997; Burton and Ingold, 1981).

1.7. Protective Role of Antioxidants on Biological Functions

Substantial research work has been carried out to investigate the preventive role of antioxidants in different diseases. Every antioxidant has some significance and the best protection against oxidative stress comes from the presence of a wide assortment of interrelated antioxidants and their cofactors. The function of each particular antioxidant depends on which type of oxidative stress is imposed (Percival, 1998).

Lipid peroxidation can damage low density lipoprotein (LDL) particles in several ways. *In vitro* studies have demonstrated that lipoxygenase, superoxide anion, peroxynitrite and myeloperoxidase can oxidize LDL (Yla-Herttuala et al., 2000) which can lead to heart diseases. Studies showed that antioxidants may protect against coronary heart diseases (CHD) (Chow, 2001; Parthasarathy et al., 1999; Diaz et al., 1997). Vitamins have been shown to reduce the susceptibility of LDL to oxidation and are also known to be involved in elevating the levels of protection factors like HDL- cholesterol. Studies suggest that vitamin C may reduce the risk of hypertension (Noguchi and Niki, 2000). In addition, a high intake of vitamin C appears to protect against gastric cancer, probably through scavenging ROS formed in the gastric mucous (Woodford and Whitehead, 1998).

Further investigations on vitamin C have proved its protective effects on inhibition of tumor promotion (Lee et al., 2002).

Dietary antioxidants are important to maintain good health. Vegetables and fruits contain a variety of important nutrients that are essential for the normal functioning of the body. Tomato intake (the main source of lycopene) has been found to be associated with a lower risk of a variety of cancers in several epidemiological studies (Agarwal and Rao, 2000). Epidemiological investigations on vitamin E suggested that it may protect against the occurrence of Parkinson's disease. Some researchers have also shown that Vitamin E intake can slow down the progression of Alzeimer's disease (Vatassery et al., 1999). It was reported that supplementation with Vitamin C and E, mixed with other antioxidants, can reduce symptoms of oxidative stress during exercise (Clarson and Thompson, 2000).

Flavonoids are polyphenols and they are abundantly found in fruits, vegetables, grains, bark, roots, stems, flowers, and tea. They possess anti-inflammatory, antiallergic, antiviral and anticarcinogenic properties due to their antioxidant potential (Nijveldt et al., 2001; Hollman et al., 1996). The capacity of flavonoids to act as antioxidants depends upon their molecular structure. The position of hydroxyl groups and other features in the chemical

structure of flavonoids are important for their antioxidant and free radical scavenging activities. Quercetin, the most abundant dietary flavonol, is a potent antioxidant because it has all the right structural features for free radical scavenging activity.

Tea is found to be particularly rich in antioxidants. It exerts beneficial effects on the regulation of blood cholesterol levels. Green tea flavonoids are potent antioxidant compounds, thought to reduce incidence of cancer and heart disease.The major flavonoids in green tea are the kaemferol and catechins (catechin, epicatechin, epicatechin gallate, and epigallocatechin gallate (EGCG). Catechin has been found to regulate blood pressure and help to lower sugar levels (Ramarathnam et al., 1995).

The mechanism of action of various antioxidants against degenerative diseases is given in Table 1.2.

Table 1.2: Mechanism of action of various antioxidants against different disease

Compound	Pathology	Mechanism of action	References
Catalase	Cancer, diabetic retinopathy	Destroys hydrogen peroxide in high concentration by catalyzing its two-electron dismutation into oxygen and water	Schonbaum and Chance (1976)
Glutathione peroxidase (GPx)	Neurodegerative diseases	Catalyse the reduction of hydroperoxides at the expense of GSH.In this process, hydrogen peroxide is reduced to water whereas organic hydroperoxides are reduced to alcohols	Ursini et al. (1995)
Superoxide dismutase (SOD)	Neurodegenerative diseases	Catalyse the one-electron dismutation of superoxide into hydrogen peroxide and oxygen	Fridovich (1997)
Carotenoids	Cancer, diabetic retinopathy, chronic inflammation	Mainly act as physical quenchers of reactive oxygen	Sundquist et al. (1994)
α -Tocopherol	Cancer, neurodegenerative diseases, chronic inflammation	Donation of hydrogen atom	Burton and Ingold (1981)
Phenolics	Cancer, diabetic retinopathy, chronic inflammation	Inhibit the oxidation of lipids, fats, and proteins (RH) by donation of a phenolic hydrogen atom to the free radical	Arouma et al.(1994)
Tannins	Cardiovascular disorders	Tannins are known to enhance synthesis of nitric oxide and relax vascular segments precontracted with norepinephrine	Dwivedi (2007)

1.8. Some Common Natural Antioxidants

There are hundreds of antioxidants of natural and synthetic origin. Increasing interest in such compounds is due to their effective role against the destructive actions of free radicals. Several studies have focused on identifying different classes of antioxidants. Some important antioxidants are described below:

Vitamin E is the most common naturally occurring antioxidant. Its structure is closely related to phenolic benzochroman derivatives. It has a phytyl side chain attached to its chromanol nucleus. The ability of donating two electrons confers for radical scavenging activity (Packer and Cadenas, 2002).

Ascorbic acid (vitamin C) is a water soluble electron donor vitamin. To act as an antioxidant, it donates two electrons from the C-2 and C-3 double bond carbons, which results in the formation of an intermediate free radical, semidehydroascorbic acid. The resulting ascorbate free radicals readily reduce to a neutral ascorbate molecule (Feri et al., 1989; Packer and Cadenas, 2002a).

Carotenoids are a large group of compounds with diverse structural features. The basic skeleton of carotenoids consists of a polyisoprenoid C_{40}

carbon chain with a number of conjugated double bonds. Due to the presence of high conjugation, effective delocalization of electrons can occur along the entire length of the polyene chain.This distinctive character of carotenoids make them effective as singlet oxygen quenchers. β – carotene is bicyclic in nature with β- ionone rings at both ends of the molecule (Packer and Cadenas, 2002b).

Phenolic phytochemicals can be categorized into three major classes: non-flavonoid polyphenols, flavonoids, and phenolic acids. Phenolic compounds have aromatic rings containing one or more hydroxyl groups such as caffeic acid. Polyphenols contain multiple phenol rings within their structures; examples include catechin and ellagic acids. Flavonols and catechins are major components of tea (*Camellia sinesis*) and are responsible for its antioxidant properties (Wiseman et al., 1997).

Tannins are high molecular weight phenolic mixtures. All classes of phenolics possess antioxidant activities, depending on the number of hydroxyl groups present on benzene rings (Packer and Cadenas, 2002). Flavonoids have been shown to have antioxidant activities both by acting as hydrogen donors or chelating with metal ions. Their structures consist of two rings, A and C, fused with the phenyl ring B through its C-1 and C-2 of ring C (Evans et al., 1997).

1. 9. Measurement of Antioxidant Activity

A number of experimental models have been developed for the determination of antioxidant activity of different type of samples. These methods can be divided into two major categories (Vaya and Aviram, 2001):

1) Measuring the potential of a sample to donate an electron or a hydrogen atom to a specific reactive oxygen species or to any electron acceptor.

2) Measuring the ability to remove any source of oxidative initiation, e.g., inhibition of enzymes, chelation of transition metal ions and absorption of UV radiation.

Several factors influence the efficiency of an antioxidant and require consideration of its bioavailability, the site of action, its stability, its toxicity, and the type of reactive oxygen species that the antioxidant must react with. It is therefore necessary to select an appropriate method for the evaluation of antioxidant potential. The following assays are commonly used for the measurement of antioxidant activity.

A- Radical Scavenging Assays

Stable free radical species are commonly used to determine the antiradical activity of compounds. These radicals include 1, 1-diphenyl-2-picryhydrazyl radical (DPPH) (Blois et al., 1958; Lee et al., 1998) and 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS)

(Re et al., 1999). Other radical scavenging assays include superoxide anion scavenging assay (Gaulejic et al., 1999) and hydroxyl radical scavenging assay (Yoshiki et al., 1995). In most studies, either the phenazine methosulphate-NADH system (Gaulejic et al., 1999) or the xanthine/ xanthine oxidase system is used to produce superoxide anion radicals (Cos et al., 1998).

In many methods of detecting antioxidant activity, the ability of an antioxidant to stop the oxidation of polyunsaturated fatty acids (PUFA), such as linoleic acid is determined by exposing the PUFA to oxygen, light or free radical generators. The frequently used methods measuring lipid peroxidation/ peroxyl radical scavenging ability include:

- **Conjugated diene assay:** This assay is used for quantification of conjugated diene formed as a result of initial PUFA oxidation by measuring UV absorbance at 234 nm (Esterbauer et al., 1989).
- **Lipid peroxide assay:** In this system, oxidation of PUFA initiated by oxygen at allylic position of lipid results in an unstable mixture of lipid peroxides. The total amount of lipid peroxides can be detected iodometrically (El-Saadani et al., 1989).
- **Thiobarbituric acid reactive substances (TBARS) assay:** In this assay model, lipid peroxides formation is measured by the detection

of a stable product as a result of reaction of thiobarbituric acid with aldehydes, a decomposed product of lipidperoxides (Armstrong, 1998).

B- Enzyme Inhibition Assays

There are certain enzymes which produce reactive oxygen species, during reactions, these enzymes include lipoxygenases, cycloxygenases and xanthine oxidase etc. Xanthine oxidase catalyzes the production of uric acid and superoxide anion using xanthine/hypoxanthine as substrate (Richardson and Finley, 1997), whereas liopoxygenases and cycloxygenases produce lipid hydroperoxides as a result of arachidonic acid metabolism (Dailey and Imming, 1999). The inhibitors of these enzymes may decrease reactive oxygen species in biological systems (Vaya and Aviram, 2001).

C- Chelation of Transition Metals

The transition metals iron and copper are essential cofactors of several enzymes which are involved in oxygen metabolism. In biological systems, these metals are found with proteins and enzymes but when these are present in free state, they can catalyze free radical reactions. The relative chelating capacity of samples can be studied spectrophotometrically by measuring the ability to release iron ions from an iron-EDTA complex and to chelate iron ions (Acker et al., 1996).

D- Chromatographic Procedures

Lipid hydroperoxides are the major end products obtained during oxidation of PUFA. Many methods developed to date measure either primary hydroperoxides or secondary aldehydic products of lipid oxidation. High performance liquid chromatography (HPLC) is one of the useful methods in the quantitative measurement of lipid hydroperoxides. These HPLC methods employ chemiluminescence detection from lipid hydroperoxides and thiobarbituric acid (TBA) assay (Lunec and Griffiths, 2000).

E- Assays Using Molecular Probes

The experimental complexity and the limitations of directly monitoring reaction kinetics of the inhibited autoxidation of lipids have led to the development of more convenient methods in assessing the antioxidant capacity of a sample. Several colorimetric and fluorometric antioxidant capacity assays apply a radical reaction but without a chain propagation step, an essential step in lipid autoxidation.

In general, these assays apply a thermal radical generator to give a steady flux of peroxyl radicals in air saturated solution. Added antioxidants compete with probes (substrates in this case) for the radicals and inhibit or retard the probe oxidation. Assays with this feature include total radical

trapping antioxidant parameter (TRAP) assay, oxygen radical absorbance capacity (ORAC) assay, and crocin bleaching assay. These assays have the following components: (a) an azo radical initiator, normally AAPH; (b) a molecular probe (UV or fluorescence) for monitoring reaction progress; (c) antioxidant; and (d) reaction kinetic parameters collected for antioxidant capacity quantitation.

1.10. Objectives of the study

The study was undertaken with the following objectives:-

(1) To study the antioxidant activity and total phenolic content of *Thuja orientalis*, a medicinally important plant.

(2) To evaluate anticancer activity of *Thuja orientalis* on cervical cancer biopsies.

(3) To evaluate antioxidant activity and total phenolic content of the fruit extracts of *Ficus racemosa*.

(4) To evaluate antioxidant activity and total phenolic content of some medicinal herbs collected from Aligarh.

(5) To evaluate antioxidant activity and total phenolic content of edible flowers grown in Aligarh.

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CHAPTER -2

MATERIALS AND METHODS

2.1. Chemicals, Reagents and Biologicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Fluka Chemika (Switzerland), 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), calf thymus DNA, S_1 nuclease enzyme , 7,8-dihydroxyflavone, quercetin, RPMI-1640, gallic acid and β-carotene were from Sigma, St.Louis, MO. Folin- Ciocalteau reagent, xanthine, xanthine oxidase (from buttermilk), linoleic acid, L-Ascorbic acid, NBT and Bovine serum albumin was obtained from Sisco Research Laboratories, Mumbai. Ficoll-Paque was of Pharmacia (LKB Biotechnology, NJ), MTT cell viability assay kit was from R & D Systems, Caspase 3,8 and 9 activity kits were from Biomol, antibodies used were from Santa Cruz Biotechnology, CA. Colorimetric substrate z-LEHD-pNA were from Upstate Biotechnology, USA , DEVD-pNA and IETD-pNA were from Biomol. Nitrocellulose membrane was purchased from Bio-Rad. All other chemicals were of analytical grade.

2.2. Instruments and culture plates

Shimadzu UV-VIS Spectrophotometer (1240) was used for all spectrophotometric studies. Buchi rotavapor was used for vacuum evaporation and Remi R24 centrifuge was used for centrifugation. Remi Cyclomixer (CM-101 DX) was used for rapid mixing, 12-well tissue culture

plates were from Costar Corporation (Cambridge, MA), Gel documentation system was from Bio-Rad Laboratories, it was equipped with Quantity One gel analysis software, Bio Rad Model 3550 Microplate Reader was used for MTT and caspase activity assays. ECL (Electrochemiluminescence) kit was from Amersham.

2.3. Measurement of total phenolic content

The method of Saucier et al (1999) was used with slight modification, and the results are expressed as gallic acid equivalents (GAE). In each analysis, 1.58 ml of water was pipetted into cuvettes, followed by 20 µl of a standard solution, sample solution, or water and the solutions were mixed well. Then 100 µl of Folin-Ciocalteau (FC) reagent was added to each cuvette, and the solutions were mixed again. After 30 s and before 8 min, 300 µL of a 20% sodium carbonate solution was added. The solutions were left at room temperature for 2 h. Then the absorbance of the developed blue color was determined at 765 nm. The amount of light absorbed is proportional to the amount of oxidizable material present, that is, phenolic compounds. Gallic acid was used as a standard for the calibration curve. The total phenolic content is reported as gallic acid equivalents (µg) using the following linear equation based on the calibration curve:

$A = 0.0011x + 0.0025$ $R^2 = 0.9995$

Where **A** is the absorbance and **x** is the gallic acid equivalents (μg).

2.4. Assays Used To Measure Antioxidant Activity

2.4.1. Reducing power assay

It is a simple assay which determines the redox potential of the test samples as redox potential is directly related to total antioxidant capacity of a test sample.

Total reducing power was determined as described by Zhu et al. (2002). Plant extracts or compounds (varying concentrations) in 1ml of distilled water were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$]; the mixture was then incubated at 50° C for 30 min. Afterward, 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally 2.5 ml of the upper layer solution was mixed with 2.5 ml of distilled water and 0.5 ml $FeCl_3$ (0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

2.4.2. DPPH Radical Scavenging Activity

DPPH· (1, 1-diphenyl-2-picrylhydrazyl radical) is a stable free radical that can accept an electron or hydrogen radical to become a stable

diamagnetic molecule. The radical scavenging potential of the compounds is determined by measuring the decrease in absorbance at 517 nm, representing the formation of the reduced form of DPPH radical, which is yellow in color. The method was developed by Blois (1958) and since then it is the most convenient and simple method for testing antioxidant activity of various compounds and plant extracts.

DPPH radical scavenging activity was evaluated according to the method described by Nagai et al. (2003). The assay mixture contained 0.3 ml of 1.0 mM DPPH radical solution, 2.4 ml of 99% ethanol, and 0.3 ml of test sample solution of different concentrations. The solution was rapidly mixed and scavenging capacity was measured spectrophotometrically by monitoring the decrease in absorbance at 517 nm. Ascorbic acid was used as positive control while reaction mixture (DPPH radical solution) minus extract solution was taken as control. The percent (%) radical scavenging was calculated by the following equation.

$$\textbf{\% radical scavenging} = \left[\frac{Ac - As}{Ac} \right] X \ 100$$

Where A_c = Absorbance of control at 517 nm and A_s= Absorbance of sample at 517 nm.

2.4.3. Inhibition of Fe (II)-EDTA-H_2O_2 induced oxidative DNA Damage

Solution of DNA was prepared by dissolving 2 mg of calf thymus DNA (Sigma Chemical Company, St.Loius, MO) in 1 ml of 10 mM tris-HCl pH 7.4, 500 μg DNA and varying concentration of extract or compound (25-250 μg/ml) solution, 0.08 mM EDTA, 0.08 mM $FeSO_4$, 0.03% H_2O_2, 20 mM Na-ascorbate. All solutions were sterilized before use. After incubation at 37°C for 1 hour, S_1 nuclease digestion was performed as described by Rahman et al. (1989). The assay determines the acid soluble nucleotides released from DNA because of enzymatic digestion. Acid soluble nucleotides were determined colorimetrically using the diphenylamine method of Schneider (1957). To a 1.0 ml aliquot 2.0 ml of diphenylamine reagent (freshly prepared by dissolving 1gm of recrystallized diphenylamine in 100 ml of glacial acetic acid and 2.75 ml of conc. H_2SO_4 was added). The tubes were heated in a boiling water bath for 20 minutes. The intensity of blue color was read at 600 nm.

2.4.4. Inhibition of AAPH induced RBC hemolysis assay

Blood was obtained from healthy human donor and collected into heparinized tubes through the Blood Bank, J.N. Medical College, Aligarh Muslim University, Aligarh. Erythrocytes were separated from plasma and the buffy coat, and washed three times with 5 volumes of phosphate buffered

saline (PBS), pH 7.4. During every wash, erythrocytes were centrifuged at 4000 rpm for 10 min to obtain a packed cell preparation (Miki et al., 1987). After the last wash, the packed RBC was suspended in four volumes of PBS solution. RBC hemolysis was induced by AAPH, a peroxyl radical initiator (Miki et al., 1987). Addition of AAPH to the suspension of washed erythrocytes induces the oxidation of membrane lipids and proteins, resulting in hemolysis. Two ml of the erythrocyte suspension was mixed with 2 ml of PBS solution containing varying amounts of extracts or compounds, 2 ml of 200 mM AAPH in PBS was then added to the mixture. The reaction mixture was shaken gently while being incubated at 37°C for 3 h. After incubation, the reaction mixture was removed, diluted with eight vols of PBS and centrifuged at 4000 rpm for 5 min. The absorbance (A) of the supernatant fraction was recorded at 540 nm. Percent inhibition was calculated by the following equation

% Inhibition = [A_{AAPH} - $A_{EXTRACT}$] / A_{AAPH}

Where A_{AAPH} is the absorbance of AAPH at 540nm and $A_{EXTRACT}$ is the absorbance of alcohol /water extract at 540 nm.

2.4.5. Superoxide radical scavenging activity

Superoxide radical scavenging activity was assayed by NBT reduction method (Ko et al., 1998). The 495 µl assay mixture consisted of 50 mM

sodium carbonate buffer (pH 10.2), 0.1 mM xanthine and 25 M nitro blue tetrazolium (NBT). The reaction was initiated by addition of 5µl 20 nM xanthine oxidase in the presence or absence of each compound. The increase in absorbance at 560 nm was read after 5 min on a spectrophotometer (Shimadzu UV- VIS 1240). Superoxide radical scavenging activity was expressed by the degree of NBT reduction of test group in comparison with that of the control group (Keum et al., 2000).

2.4.6. β- Carotene- linoleic acid assay

In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Dapkevicius et al., 1998).

The mechanism of bleaching of β-carotene is a free radical mediated phenomenon resulting from the hydroperoxides formed from linoleic acid. β-carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated β-carotene molecules. As β-carotene molecules lose their double bonds by oxidation, the compound loses its chromophore and characteristic orange color, which can be monitored spectrophotometrically.

The presence of different extracts can hinder the extent of β-carotene bleaching by neutralizing the linoleate free radical and other free radicals formed in the system.

A stock solution of β-carotene was dissolved in 1 ml of chloroform (HPLC grade), 25 µl linoleic acid and 200 mg Tween 40 were added. Chloroform was completely evaporated using a vacuum evaporator. Then, 100 ml of distilled water saturated with oxygen (30 min, 100 ml/min) were added with vigorous shaking. 2500 µl of this reaction mixture were dispensed into test tubes and 350 µl portions of the test samples (at a concentration of 25µg/ml for plant extracts & 20 µM for compounds) were added and the emulsion system was incubated for up to 48 h at room temperature. The same procedure was repeated with the synthetic antioxidant, butylated hydroxytoluene (BHT) as positive control, and a blank. After this incubation period, absorbances of the mixtures were measured at 490 nm. Antioxidative capacities of the extracts were compared with those of BHT and blank.

2.4.7. Lipid Peroxidation assay

The inhibition of lipid peroxidation by the test samples was determined by quantification of the lipid peroxidation product malondialdehyde (MDA) based on reaction to thiobarbituric acid using egg

yolk as oxidisable substrate (Dasgupta et al., 2004). Briefly 0.5 ml egg homogenate (10% distilled water, v/v) and 0.5 ml of test sample were mixed, then 0.05 ml of $FeSO_4$ (0.07 M) was added to initiate lipid peroxidation. After incubation for 30 min, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% (w/v) thiobarbituric acid in 1.1% sodium dodecyl sulphate were added to quench the reaction, resulting mixture was vortexed and then heated at 95°C for 60 min. After cooling, 5.0 ml of n-butanol were added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm. Allocated as a positive control was BHT.

2.5. Methods used to measure Anticancer activity

2.5.1. Preparation of PBMC and cell culture

Blood was collected by venipuncture in heparinized syringes and PBMC (peripheral blood mononuclear cells) were isolated by density gradient sedimentation on Ficoll-Paque separation medium (Wilkinson et al. 2001). PBMC (5×10^6 cells/well) were added in 12-well tissue culture plates (Costar Corp., Cambridge, MA) in complete RPMI-1640 medium, and were subsequently incubated at 37°C, 5% CO_2 for 1-2 h for adherence. Non-adherent cells were washed away with RPMI medium, and adherent

monocytes were rested overnight in RPMI having 2% serum at 37°C, 5% CO_2.

2.5.2. MTT assay

This test is a quantitative colorimetric method to determine cell proliferation. It utilizes the yellow tetrazolium salt [3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide] which is metabolized by mitochondrial succinic dehydrogenase activity of proliferating cells to yield a purple formazan reaction product (Buttke, 1993).

MTT Cell Viability Assay Kit (R & D Systems) was used according to the manufacturer's instructions.

Reagents supplied in the kit:

Component	Quantity	Storage conditions
MTT reagent	25 ml	2-8 ° C
Detergent reagent	250 ml	18-24 ° C

Assay procedure

Adherent monocytes (both normal and from cervical cancer patients, n=8 for both) treated with the test samples for 24 hrs were gently scraped

with RPMI-1640 medium. After this, monocytes (3×10^4 cells/well in 100 µl) were added in 96-well tissue culture plates. Cells were incubated in RPMI-1640 with 2% autologous serum containing test samples (0-25 µg/ml) for 24 hours at 37° C, 5% CO_2. After 24 hours, 10 µl of MTT reagent (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) was added to each well and incubation was continued for an additional two hours. When a purple precipitate was clearly visible under the microscope, 100 µl of detergent was added to all wells including control wells and incubated for two hours in the dark at 20 °C. After incubation, the precipitate was solubilized and the absorbance of the resulting solution was measured at 570 nm using a microplate reader. Control cells were treated in exactly the same way except that no test samples were added to the wells. The percentage of viable cells was calculated by the formula as described by Islam et al. (2000) and the results are expressed as "Viable cells (% of control cells)".

$$\textbf{Viable monocytes (\% of control cells)} = \frac{\textbf{Absorbance value of control cells}}{\textbf{Absorbance value of treated cells}} \times \textbf{100}$$

2.5.3. Caspase 3, 8 and 9 activity assay

2.5.3.1. Caspase-3 activity assay

Caspase-3 related protease activity in cell lysates was determined using a commercially available kit (Biomol). The substrate employed was Asp-Glu-Val-Asp-p-nitroanilide (DEVD-pNA) (Biomol). Cell lysate protein (nuclei free) was mixed with assay buffer (containing 10 mmol dithiothreitol and the colorimetric substrate DEVD-pNA (50μmol) followed by incubation at 37° C for 1h. Absorbance was then read with a plate reader at 405 nm and the activity units were determined according to the instructions provided with the kit.

2.5.3.2. Caspase-8 activity assay

Caspase-8 related protease activity in cell lysates was determined using a commercially available kit (Biomol). The substrate employed was Ile-Glu-Thr-Asp-p-nitroanilide (IETD-pNA) (Biomol). Cell lysate protein (nuclei free) was mixed with assay buffer (containing 10 mmol dithiothreitol and the colorimetric substrate IETD-pNA (50μmol) followed by incubation at 37° C for 1h. Absorbance was then read with a plate reader at 405 nm and the activity units were determined according to the instructions provided with the kit.

2.5.3.3. Caspase-9 activity assay

Caspase-9 related protease activity in cell lysates was determined using a commercially available kit (Biomol). The substrate employed was Ac-Leu-Glu-His-Asp-p-nitroanilide(z-LEHD-pNA) (Upstate Biotechnology, USA). Cell lysate protein (nuclei free) was mixed with assay buffer (containing 10 mmol dithiothreitol and the colorimetric substrate zLEHD-pNA (50μmol) followed by incubation at 37° C for 1h. Absorbance was then read with a plate reader at 405 nm and the activity units were determined according to the instructions provided with the kit.

2.5.4. PARP cleavage and Western blot analysis

At the end of treatment, cells were suspended in ice cold RIPA-M buffer with 1% Nonidet P-40 and cell lysate prepared as described earlier (Islam et al., 2000). Cell lysate protein (30μg) was resolved by gradient (4-20%) SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). The blots were blocked for at least 1h at room temperature in blocking buffer (5% non fat dry milk in Tris-buffered saline containing 0.05% Tween 20). Primary anti-PARP antibody was diluted in blocking buffer and incubated with the blots overnight at 4°C. The blots were washed three times with TBST (Tris- buffered saline and 0.05 % Tween 20) and the bound antibody detected with a 1:5000 dilution of HRP-conjugated sec

antibody following the instructions provided with the ECL kit (Amersham). Blots were stripped and reprobed with anti-β-Actin antibody (Santa Cruz Biotechnology, CA) and detected by enhanced chemiluminescense detection system (ECL, Amersham Corporation, UK).The band was quantified by Scion Image (Scion Corporation, USA) and expressed as area × density.

2.5.5. Studies on cervical biopsies

Biopsy pieces were taken directly from untreated cervical-cancer patient (n=8) admitted for the first time and showing frank fungating growth. The study utilizing biopsies and blood collection was approved by the Institutional Committee. Biopsies showing differentiated keratinizing and necrotising lesions were excluded from the study. Eight selected cervical biopsies maintained in RPMI-1640 medium, were each divided into two parts and kept in two separate vials containing 1ml of RPMI-1640 medium with 2 mM HEPES buffer and 2% autologous serum. One vial was treated with test sample and taken as control. They were then incubated for 24 hours at 37°C.

The vials were then sent to the Cytopathology Laboratoty, Department of Pathology, J.N. Medical College, AMU, Aligarh. 0.5 ml of the supernatant from the culture medium suspension after shaking non-vigorously for 10 minutes was taken and centrifuged at 500 rpm for 5

minutes using Shandon Cytospin 4. The cytocentrifuged smears were stained using Haemotoxylin & Eosin stain and Papanicolaou stain. The remaining tissues in test and control vials were added 1 ml of RPMI-1640 culture medium with test sample and without test sample (control), incubated for next 24 hours and processed for cytocentrifuged smears.

2.5.5.1. Staining Methods

- **Haematoxylin and Eosin Method**

The staining was done by a modification of the method described by Culling et al (1985) as used in our cytology laboratory.

Reagents

Harris' Haematoxylin

· Harris' Haematoxylin	5.0 gm
· Absolute alcohol	50.0 ml
· Alum (ammonium/potassium)	100.0 gm
· Distilled water	1000.0 gm
· Mercuric Oxide	2.5 gm

Eosin

· Eosin Y	16 gm
· Potassium dichromate	8 gm
· Picric acid	160 ml
· 95% alcohol	160 ml
· Distilled water	1280 ml

Procedure

After removing from the fixative the smears were passed through descending alcohol grades (65%, 80%, 70%, 50% ethyl alcohol) to rehydrate the smears. The smears were then left under tap water for 10 minutes, following which they were immersed in Harris' Haematoxylin for 2 minutes. They were then washed in running tap water for 1 minute to remove the excess stain. The smears were then dipped in 1% acid alcohol (2-3 dips), following which they were washed in running tap water for 1 minute. The smears were then dipped in Eosin solution for 20 seconds followed by washing in running tap water for 1 minute. The smears were then dehydrated by dipping in 95% ethanol and absolute alcohol (15 dips in each). Clearing was done in xylol (15 dips). The slides were mounted in DPX (Distrene dibutylphthalate xylol), after drying.

Result: Cytoplasm – pink

Nucleus – blue

- **Papanicolaou stain**

Reagents

Harris' Haematoxylin (without acetic acid)

Orange G (OG6)

*	0.5 OG6 in 95% alcohol	100 ml
*	Phosphotungstic acid	0.015 gm

Eosin alcohol (EA36)

*	0.5% Light green SF	45 ml
*	0.5% Bismarck brown	10 ml
*	0.5% Eosin Y	45 ml

Phosphotungstic acid 0.2 gm

Procedure

The staining was done by a modification of the method described by Culling et al (1985) as used in our Cytopathology Laboratory.

Alcohol fixed smears were rehydrated by dipping them sequentially in decreasing grades of ethyl alcohol (80%, 70%, 50%) and then in distilled water (10 dips in each). The smears were then placed in Haris' Haematoxylin for 6 minutes, following which they were rinsed twice in water (10 dips each). The smears were then differentiated in 0.25% HCl (6

dips). Blueing was done in tap water for 6 minutes. The smears were then dipped sequentially in ascending grades of ethyl alcohol (50%, 70%, 80%, 95% - 10 dips each). The smears were then dipped in Orange G-6 for 1-2 minutes, following which they were dipped in 95% alcohol (3 changes of 10 dips each). The smears were then dipped in Eosin alcohol 36 for 1-2 minutes, following which they were dipped in 95% alcohol (3 changes of 10 dips each). The smears were then dipped in absolute alcohol (3 changes of 10 dips each). Clearing was done in xylol (3 changes of 10 dips each). The slides were mounted with DPX, after drying.

Results: Acidophilic cells and Keratin - red to orange

Basophilic cells - green to blue green

Nuclei - blue

Red blood cells - orange

The remaining tissues, test and control at 48 hours were fixed in buffered formol saline. After fixation, the tissues were then processed in the histokinete, then they were transferred to a mould (Leuckhart's L pieces) filled with molten wax. Long ribbons of thin paraffin sections (3-5 u in thickness) were cut by rotatory microtome (Shandon Finesse 315). Staining was done by haematoxylin and Eosin stain.

- **Haematoxylin and Eosin staining:**

The staining was done by a modification of the method described by Culling et al (1985), as used in our Histopathology Laboratory.

Procedure:

The slides were dewaxed by application of heat and were placed in xylol for 1-2 minutes. Hydration was done by graded alcohol (absolute alcohol, 90%, 70%, 50%). The slides were then washed in running tap water for 1-2 minutes, followed by immersion in Harris' Haematoxylin for 10 minutes. The slides were then again washed in running tap water for 5-10 minutes. Decolourisation was done using 1% acidic alcohol, following which the slides were rinsed in running water for 30 seconds. Counterstaining was done with 1% Eosin for 2-4 minutes, followed by washing in running water to wash off the excess stain. The slides were then dehydrated by dipping in 95% ethanol and absolute alcohol (15 dips in each). Clearing was done in xylol (15 dips) and slides were mounted with DPX, after drying.

Results: Nucleus - blue

Cytoplasm - pink

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CHAPTER-3

ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC CONTENT OF THUJA ORIENTALIS

3.1. INTRODUCTION

Thuja orientalis L. [syn. *Platycladis stricta* Spach; *Platycladis orientalis* (L.) Franco; *Biota orientalis* (L.) Endl.], commonly known as Oriental Thuja, Oriental Arbor Vitae and Chinese Arbor Vitae, is an ornamental conifer of the cypress family. The name 'Arbor vitae' is from Latin, 'tree of life', and is related to long life and vitality in Buddhist thought in China.

Biological activity: The leaves of *Thuja orientalis* L [Syn. *Biota orientalis* (L.) Endl. (Cupressaceae)] have been used in Chinese medicine for the treatment of rheumatism, diarrhoea and chronic tracheitis (Jiangsu College of New Medicine, 1977). Some of the ethnobotanical uses and medicinal properties attributed to *Thuja occidentalis* (Grieve, 1994) are also attributed to *Thuja orientalis*. For example, *Thuja orientalis* is a nerve stimulant, expectorant, diuretic, astringent and counter irritant, and the plant extract can be used to treat warts and bronchitis with cardiac weakness (British Herbal Pharmacopoeia, UK, 1983; Mabey, R., 1988). The volatile oil of *Thuja orientalis* has shown antimicrobial properties (Bagci and Digrak, 1996). Dietary *Thuja orientalis* seed oil suppresses anti-erythrocyte autoantibodies and prolongs survival of NZB mice (Lai et al., 1994). Platelet activating factors, receptor binding antagonists have also been isolated from

Thuja orientalis, and out of six compounds isolated, cedrol and pinusolide were found to be active (Yang et al., 1995). The *Thuja orientalis* extract, quercetin and rutin, reduced serum urate levels of hyperuricemic mice caused by oxonate (Zhu et al., 2004). The compounds isolated from the leaves inhibit expression of adhesion molecules induced by tumor necrosis factor-α on inflammatory cells (Lee et al., 1999).

Chemistry: The occurrence of terpenoids (Tomita et al. 1969a; Tomita et al. 1969b; Tomita and Hirose 1969c), flavonoids (Natarajan et al., 1970; Pelter et al., 1970; Lee et al., 1999) and coumarin (Lee et al., 1999) in various parts of *T. orientalis* has been reported.

3.2. MATERIALS AND METHODS

3.2.1. Plant material

The leaves of *Thuja orientalis* was collected in the month of March from the University Garden, Aligarh Muslim University, Aligarh (U.P.), India and identified by Dr. Athar Ali Khan, Department of Botany, A.M.U., Aligarh and the voucher specimen has been deposited in the herbarium of Department of Wildlife Sciences, A.M.U., Aligarh (Voucher No. 940).

3.2.2. Preparation of the extracts

Dried and powdered leaves of *Thuja orientalis* (50g each) were extracted with ethanol and water, separately. The extracts were dried under reduced pressure at 40°C and used for various studies.

3.2.3. Determination of total phenolic content

Total phenolic content was determined by Folin-Ciocalteau Method. It is described in detail in Chapter 2.

3.2.4. Antioxidant activity measurement

Antioxidant activity was measured by determining the reducing power, DPPH radical scavenging, inhibitory effect on Fenton reaction induced oxidative DNA damage and inhibition of RBC hemolysis induced by AAPH. The procedures are described in detail in Chapter 2.

3.3. RESULTS

3.3.1. Yield of the extracts

The evaporation in vacuum yielded crude ethanol extract (8.2g, 16.4%) and aqueous extract (4.5g, 9%).

3.3.2. Amount of total Phenolic Compounds

As shown in Table 3.1, both water and alcohol extracts of *T. orientalis* have good amount of phenolic compounds. The water extract has greater amount of total phenolics as compared to alcohol extract.

Table 3.1: Amount of total phenolic compounds in water and alcohol extracts of *Thuja orientalis*.

Extracts (200 µg/ml)	Absorbance (760 nm)	Gallic acid equivalents (µg/200 µg of dry weight)
Water extract	0.0724	63.54
Alcohol extract	0.0550	47.727

3.3.3. Reducing power

The reducing power determined by the present assay depends on the redox potentials of the compounds present in water and alcohol extract of *Thuja orientalis*, characterized by the complexity of their constituents. As shown in Figure 3.1, reducing power of water extract was higher than alcohol extract.

3.3.4. DPPH Radical Scavenging Activity

As shown in Figure 3.2, DPPH decolorization was increased by the *T. orientalis* extracts in a concentration dependent manner. Both water and alcohol extracts are good scavengers of DPPH free radical. Water extract of *T. orientalis* at a concentration of 500 µg/ml was able to scavenge 68.59% of DPPH free radical while alcohol extract at the similar concentration was able

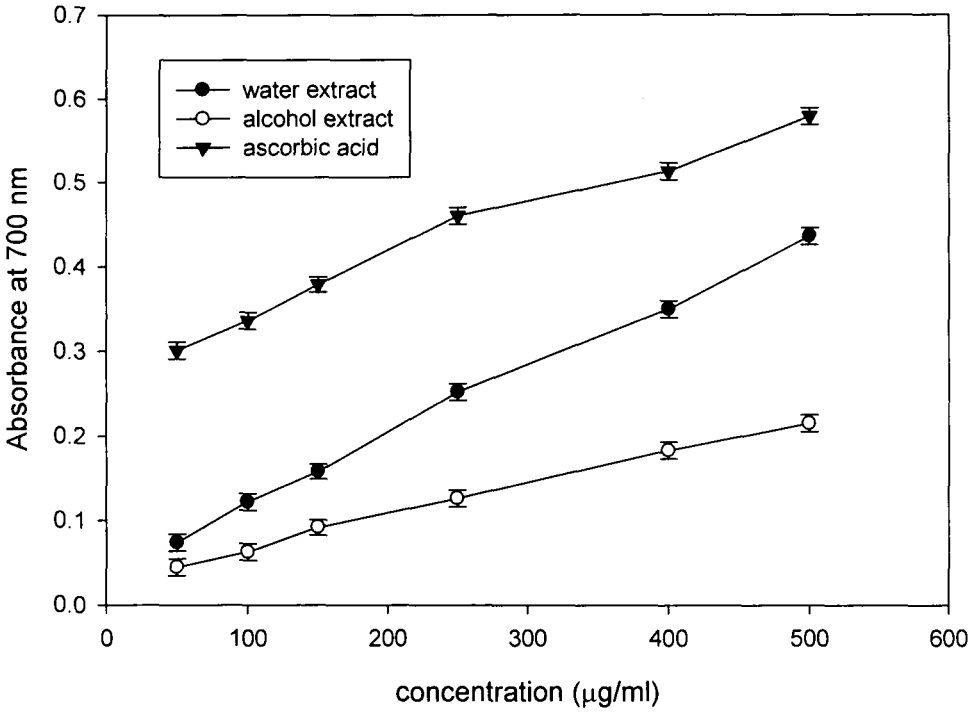

Figure 3.1: Reducing power of water and alcohol extracts of *Thuja orientalis.* Ascorbic acid is used as a standard. Values are expressed as Mean ± S.E.M., n=3.

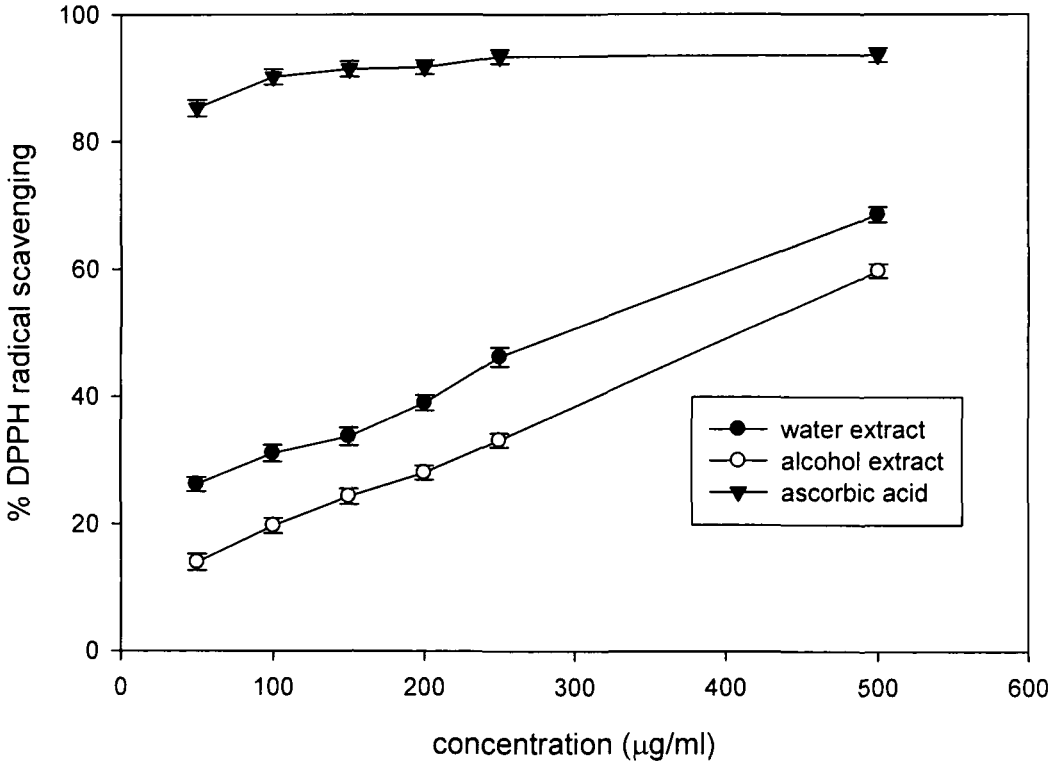

Figure 3.2: Percent DPPH radical scavenging activity of water and alcohol extracts of *Thuja orientalis*.Ascorbic acid is used as a standard.Values are expressed as Mean ± S.E.M., n=3.

to scavenge 59.73% of DPPH free radical. Thus, water extract is a better scavenger of DPPH free radical as compared to alcohol extract.

3.3.5. Inhibition of Fe (II)-EDTA-H_2O_2 induced oxidative DNA Damage

We studied alcohol and water extract of *T.orientalis* for their ability to modulate DNA damage produced by Fenton reaction. Control experiments (not shown) established that heat denatured DNA underwent 100% hydrolysis following treatment with S1 nuclease, whereas native DNA resulted in <10 % conversion. S_1 nuclease hydrolysis of DNA decreases in a dose dependant manner with the increasing concentration of water and alcohol extracts of *Thuja orientalis* (Figure 3.3). At a concentration of 250 µg/ml, water and alcohol extract of *T.orientalis* inhibited the hydrolysis of DNA by 72.859% and 65.312%, respectively while standard Quercetin inhibited DNA hydrolysis by 85.34% (Figure 3.4).

It shows that water extract is a stronger inhibitor of DNA cleavage, because at all concentrations tested, it inhibits DNA degradation to a greater extent than the alcohol extract. Thus, *T. orientalis* extracts were able to prevent DNA hydrolysis and oxidative damage to DNA.

3.3.6. RBC Hemolysis

Lipid oxidation of human erythrocytes (RBC) membrane mediated by AAPH induces membrane damage and subsequently hemolysis (Miki et al.

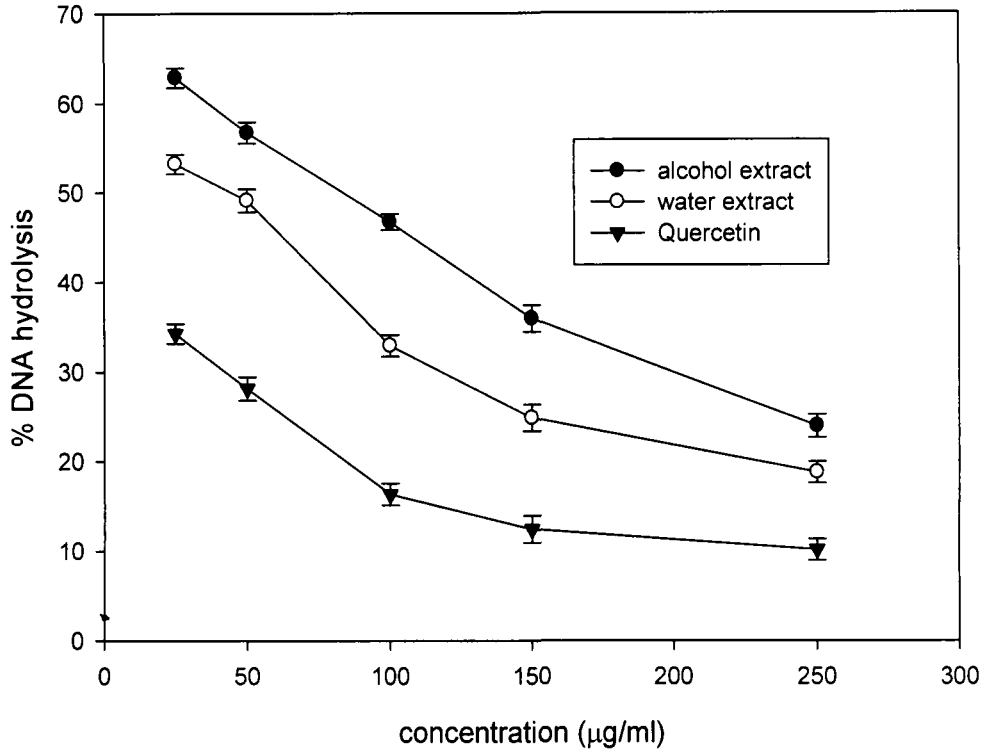

Figure 3.3: Decrease in degradation of DNA in the presence of water and alcohol extracts of Thuja orientalis.Quercetin is used as a standard. Values are expressed as Mean ± S.E.M., n=3.

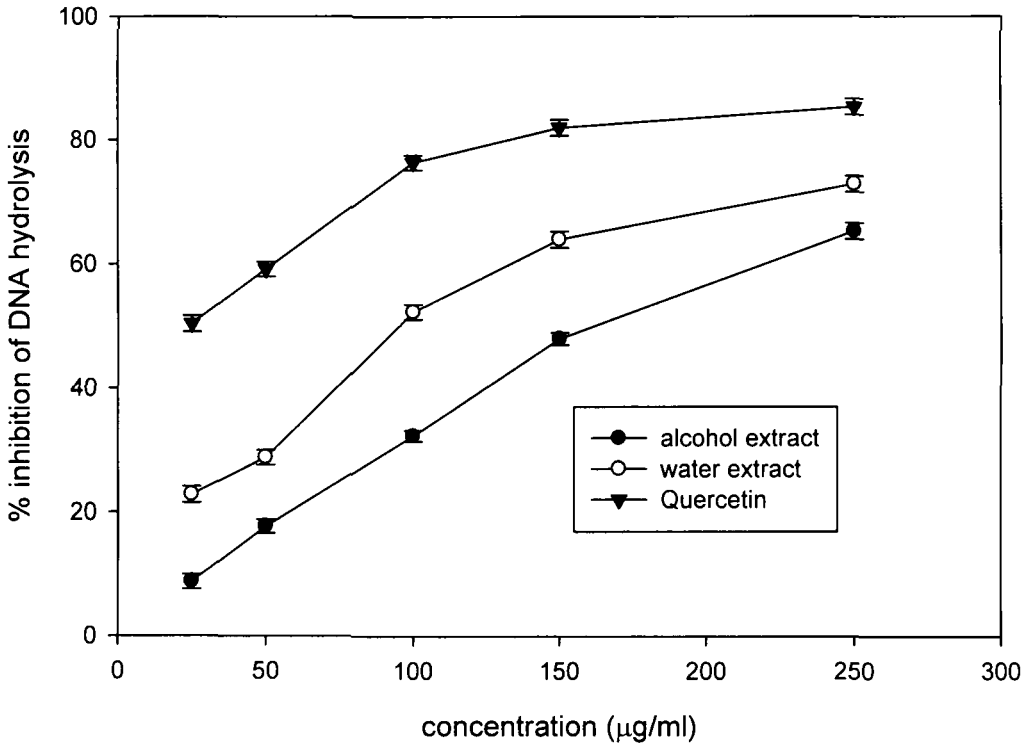

Figure 3.4: Inhibition of DNA hydrolysis by water and alcohol extracts of *Thuja orientalis*.Quercetin is used as a standard. Values are expressed as Mean ± S.E.M., n=3.

1987). Both water and alcohol extracts of *Thuja orientalis* showed inhibition of RBC hemolysis. As shown in Table 3.2, at a concentration of 12.5 µg/ml, water and alcohol extracts inhibited RBC hemolysis to the extent of 69.30% and 54.55%, respectively. Both extracts demonstrated dose-dependent inhibition effects toward RBC hemolysis, with the protective effect, reaching maximum at a concentration of 50 µg/ml.

Table 3.2: Inhibitory Effect (%) of water and alcohol extracts of *Thuja orientalis* on AAPH Induced Hemolysis of Human Red Blood Cells*.

Compound/extract	12.5µg/ml	25µg/ml	50µg/ml
Ascorbic acid	80.44 ±0.42	87.76 ±0.75	92.38 ±0.80
Water extract	69.30 ±0.25	75.15 ±0.84	88.14 ±1.09
Alcohol extract	54.55 ±0.66	68.34 ±0.92	79.21 ±1.31

*Data are expressed as Mean ± S.E.M. (Standard error of mean), n = 5.

3.4. DISCUSSION

In the present study, water and alcohol extracts of *Thuja orientalis* showed antioxidant activity and protected DNA and human red blood cell from free radical induced damage. Since free radicals are involved in the etiology of several degenerative diseases and various inflammatory diseases (Ames et al., 1993), thus leaf extracts of *Thuja orientalis* which are showing

significant antioxidant activity, might be helpful in slowing the progress of various oxidative stress-related diseases.

Our findings further support the use of *Thuja orientalis* in Chinese medicine for the treatment of rheumatism, gout, diarrhoea and chronic tracheitis (Jiangsu College of New Medicine, 1977), because oxygen free radicals have also been implicated as mediators of tissue damage in patients with rheumatoid arthritis (Cimen et al., 2000; Thabrew et al., 2001; Taysi et al., 2002; Karatas et al., 2003).

Our study showed that the reducing capacity of water extract is higher than the alcohol extract. The greater reducing power of water extract might be due to presence of water-soluble polyphenols and flavanol glycosides, which are extracted in greater amount in the water extract.

The antioxidant activity of *Thuja orientalis* extracts was determined by DPPH radical scavenging ability. This method is based on the reduction of DPPH, a stable free radical. Because of the odd electron of DPPH, it gives a strong absorption maximum at 517 nm by visible spectroscopy (purple color). As the odd electron of the radical becomes paired off in the presence of a hydrogen donor, that is, a free radical scavenging antioxidant, the absorption strength decreases, and the resulting decolorization is stiochiometric with respect to the number of electron captured (Blois, 1958).

This reaction has been widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate the antioxidative activity of foods and plant extracts (Ekanayake et al., 2004; Zhou et al., 2004; Panovska et al., 2005; Rodriguez et al., 2005). The water extract showed higher DPPH radical scavenging ability as compared to the alcohol extract. Better scavenging ability of water extract as compared to alcohol extract can be attributed to greater amount of phenolic compounds in water extract as shown in Table 3.1. It is further supported by the presence of flavonoid glycoside (Khabir et al. 1985) and condensed tannin (Zhenwen et al., 1983), which are highly soluble in water and are easily extracted in water.

The oxidative stress due to oxygen and various radical species is associated with the induction of DNA single and double strand breaks and is considered to be the first step in several human degenerative diseases, such as, cancer and ageing (Lieber, 1998). In the Fe (II)-EDTA-H_2O_2 induced oxidative DNA damage assay both water and alcohol extract protected DNA from hydroxyl radical induced damage. At a concentration of only 250 µg/ml, water extract inhibited DNA hydrolysis by 72.859 %. Alcohol extract also showed good protective ability in this assay and at a concentration of 250 µg/ml, inhibited DNA hydrolysis by 65.312 %. This is attributable to

the presence of flavonoid glycosides (Khabir et al. 1985), flavones (Yang et al. 1995), biflavones (Natarajan et al., 1970) and condensed tannin (Zhenwen et al., 1983) with known chemopreventive properties (Hertog et al., 1993; Chung et al., 1998; Aherne and O'Brien, 2000). Therefore, *T. orientalis* extracts are potential chemopreventive agents.

It is well recognized that the oxidation of polyunsaturated fatty acids in biological membranes can lead to the formation and propagation of lipid radicals, uptake of oxygen, rearrangement of the double bonds in unsaturated lipids, and even destruction of membrane lipids. Many of these biochemical activities can lead to the production of breakdown products that are highly toxic to most cell types. Excessive oxidative damage to cellular membranes contributes to the initiation and progression of numerous degenerative diseases, including certain cancers and cardiovascular disease (Pryor, 2000; Young and Woodside, 2001). Red blood cells are vulnerable to lipid peroxidation due to their high content of polyunsaturated lipids, their rich oxygen supply, and the presence of transition metals. Lipid oxidation of human red blood cell membrane mediated by AAPH induces membrane damage and subsequently hemolysis (Miki et al., 1987; Zhu et al., 2002). *Thuja orientalis* extracts inhibited AAPH (an azo peroxyl radical initiator) mediated human red blood cell hemolysis. At a concentration of 12.5 μg/ml,

water and alcohol extracts inhibited RBC hemolysis to the extent of 61.30% and 54.55%, respectively. At a concentration of 50 μg/ml, water extract inhibited RBC hemolysis by 88.14%, which is only 4 % less than L-ascorbic acid at similar concentration. Alcohol extract at the similar concentration showed inhibition by 79.21%, which is 9% less than water extract. This may again be attributable to higher amount of polyphenolic compounds in water extract. Thus, there is direct relation between amount of phenolic content and antioxidant activity. Since *Thuja orientalis* extracts showed protection against lipid peroxidation of RBC membrane; it can also be used in the prevention of cardiovascular diseases.

In conclusion, the data reported in the present study demonstrates that water and alcohol extracts of *Thuja orientalis* have free radical scavenging activity and reducing power, and can provide protection against DNA oxidation and RBC hemolysis. Therefore, the herbal formulations based on *Thuja orientalis* extracts can be used for the prevention and treatment of oxidative stress related disorders, such as, cancer, vascular diseases, gout and rheumatism.

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CHAPTER – 4

ANTICANCER ACTIVITY OF THE LEAF EXTRACTS OF THUJA ORIENTALIS

4.1. INTRODUCTION

Cervical cancer is the second most common malignant neoplasm in women, in terms of incidence and mortality rates worldwide. This involves expression of both pro- and anti-apoptotic proteins that have varied effect on tumor growth and metastatis.

Chemopreventive agents are of great interest since they may reduce the incidence of cancer in human populations. Several new anticancer agents that entered the market in the 1990s were obtained from natural sources. There are also a significant number of naturally derived new anticancer candidate compounds that are currently undergoing preclinical and early clinical development (Cragg and Newman, 1999).

Thuja orientalis is an important medicinal plant. The leaves of *Thuja orientalis* L [Syn. *Biota orientalis* (L.) Endl. (Cupressaceae)] have been used in Chinese medicine for the treatment of rheumatism, diarrhoea and chronic tracheitis (Jiangsu College of New Medicine, 1977). The water and alcohol extracts of the leaves of *Thuja orientalis* have been shown to possess antioxidant activity (Nizam and Mushfiq, 2007).In this study, the anticancer activity of the leaf extracts of *Thuja orientalis* is studied on cervical cancer cells.

4.2. MATERIAL AND METHODS

4.2.1. Preparation of the leaf extracts

The preparation of extracts is described in detail in Chapter 3.

4.2.2. Preparation of PBMC and cell culture

The procedure is described in detail in Chapter 2.

4.2.3. MTT cell viability assay

The detailed procedure is given in Chapter 2.

4.3. RESULTS

4.3.1. MTT assay

To assess whether the water and alcohol extracts of *Thuja orientalis* inhibited the growth and proliferation of cervical cancer cells, cells were treated with various concentrations of test sample (2-25 μg/ml) and the cell viability was determined by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay. The results obtained with the MTT assay are shown in Figure 4.1 and the cell viability is expressed as percent mean ± standard deviation (S.D.) viable cells compared to untreated cells (taken as 100% viable) at different concentrations of the test samples. Both the extracts inhibited the growth of cervical cancer cells in a dose dependent manner. Alcohol extract of the leaves of *Thuja orientalis* (TO-al) and 7,8-

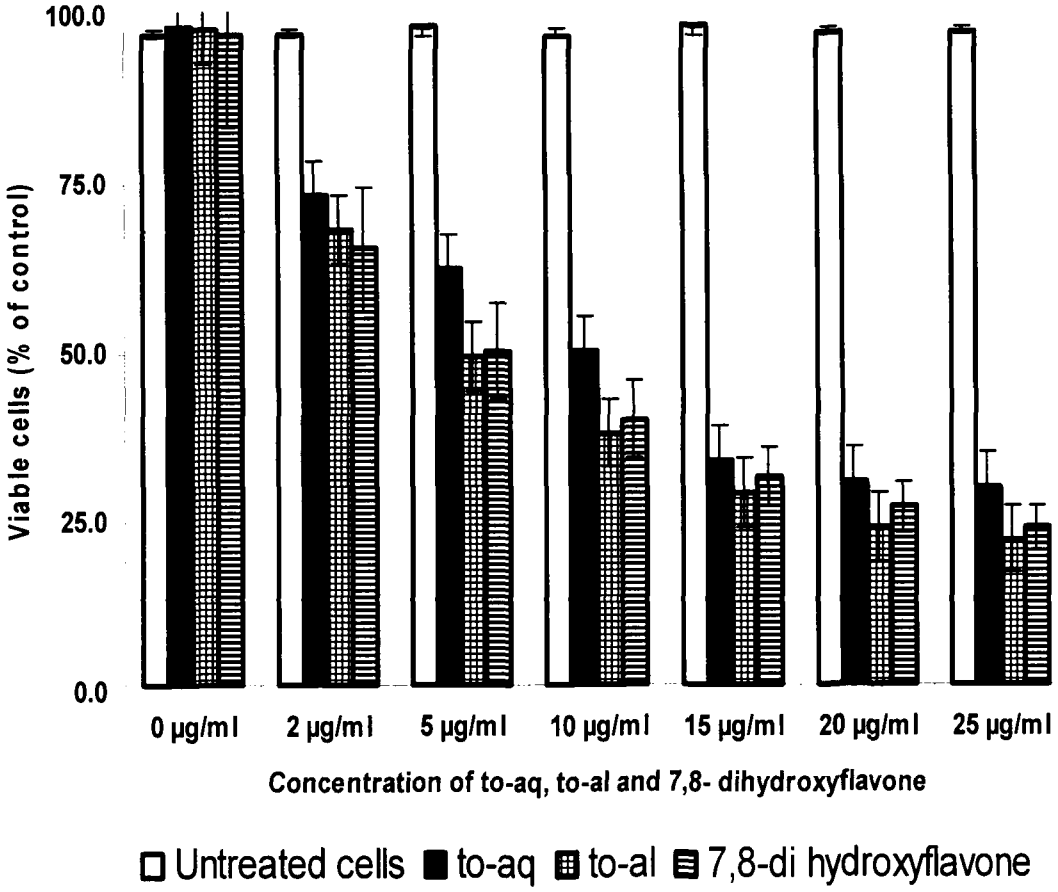

Figure 4.1: Determination of cell viability by MTT assay. Values are expressed as Mean $\pm$ S.D., n=10

dihydroxyflavone (positive control) were potent inhibitor of cell proliferation and the cell viability was around 28% and 31% after 24 hrs of treatment with 5 μg/ml of TO-al and 7,8-dihydroxyflavone respectively (Figure 4.1). Based on this data ($P < 0.001$ for all), TO-al and 7, 8-dihydroxyflavone were used at a concentration of 5μg/ml in subsequent experiments as this concentration produced high magnitude inhibition in cell proliferation when compared to controls.

4.3.2. PARP cleavage and Western blot analysis

Treatment with TO-al induced apoptosis and Poly (ADP-ribose) polymerase (PARP) cleavage in cervical cancer cells (Figure 4.2A, 4.2B) while there is no apparent effect of the treatment of TO-aq on the cleavage of PARP. Apoptotic cell death is characterized by chromatin condensation, membrane blebbing, intranucleosomal fragmentation of DNA, activation of caspases, and apoptotic body formation. An important feature of apoptotic cell death is the activation of caspase-3 like proteases in response to death inducing stimuli resulting in the cleavage of PARP producing a fragment at ~ 85 kDa. This causes the loss of normal PARP function which irreversibly commits the cell to die (Tewari et al., 1995; Orth et al., 1996). To determine whether TO-al and 7,8-dihydroxyflavone induced apoptosis in cervical cancer cells were grown to confluence (80-90%) and treated with TO-al and

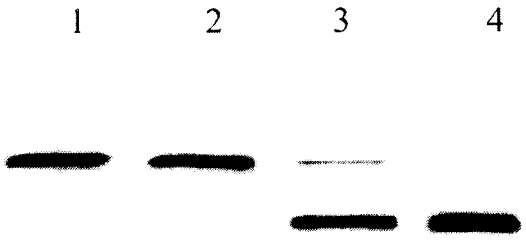

Figure 4.2 A: PARP- cleavage
Lane 1 = untreated control cancer cells.
Lane 2 = cancer cells treated with TO-aq.
Lane 3 = cancer cells treated with TO-al.
Lane 4 = cancer cells treated with 7, 8-dihydroxyflavone.

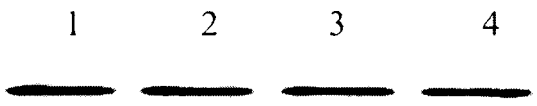

Figure 4.2 B: β-actin check.
Lane 1 = untreated control cancer cells.
Lane 2 = cancer cells treated with TO-aq.
Lane 3 = cancer cells treated with TO-al.
Lane 4 = cancer cells treated with 7, 8-dihydroxyflavone.

7,8-dihydroxyflavone (5 μg/ml or 5μM) for 24 h. The test sample containing mediums were then replaced with fresh medium and the cells allowed to grow overnight. Cells were washed and the cell lysate prepared and analyzed for PARP cleavage as an indicator of caspase activation by Western blotting. The antibody used (clone 7D3-6) recognizes the intact 116 kDa and the cleaved 85 kDa fragments. Previous studies have shown that the pattern of PARP cleavage differs in necrotic and apoptotic cells (Shah et al., 1996) and, thus, this assay can be used to determine whether treatment with a particular agent induced apoptosis or necrosis. The results shown in Figure 4.2 A clearly show the presence of the ~ 85 kDa fragment of PARP in TO-al and 7, 8-dihydroxyflavone treated cultures but not in the aqueous extract of *Thuja orientalis* (TO-aq) and untreated control cultures.

4.3.3. Caspase 3, 8 and 9 activities

The activity of caspase-3, 8 and 9 in TO-aq, TO-al and 7,8-dihydroxyflavone (7,8-DHF) treated cells was also measured. The results (Figure 4.3, 4.4 and 4.5) showed that cells treated with the extracts of *Thuja orientalis* and 7, 8-dihydroxyflavone had significantly higher activity of caspase-3 and caspase-8 like proteases indicating that activation of proteases was associated with reduced cell survival and apoptotic death of treated cervical cancer cells. To test this hypothesis, we pre-treated the cervical

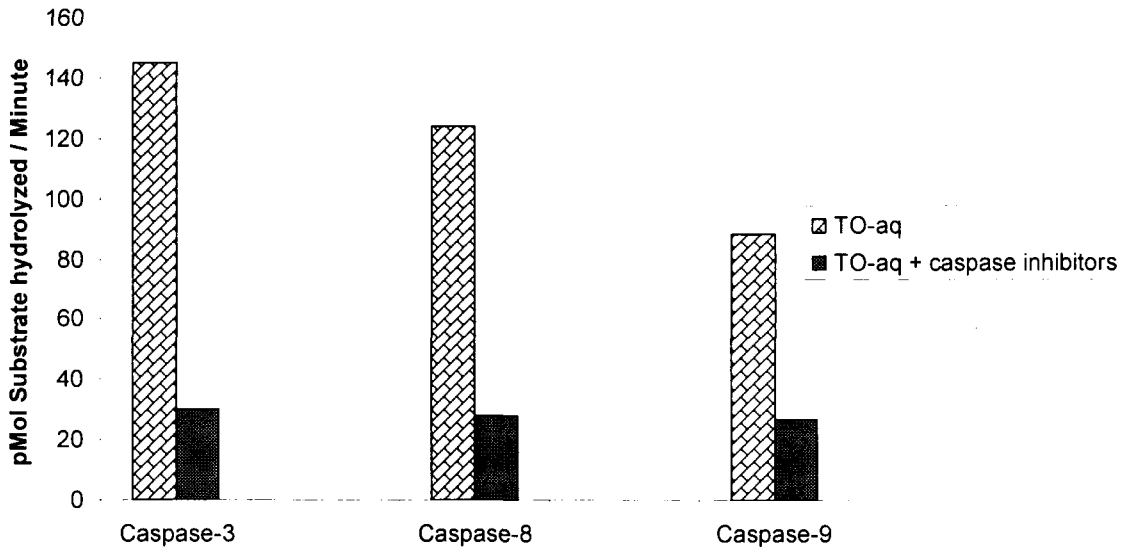

Figure 4.3: Caspase 3,8 and 9 activities of the aqueous extract of *Thuja orientalis* (TO-aq).Values are expressed as Mean ± S.D., n=8.

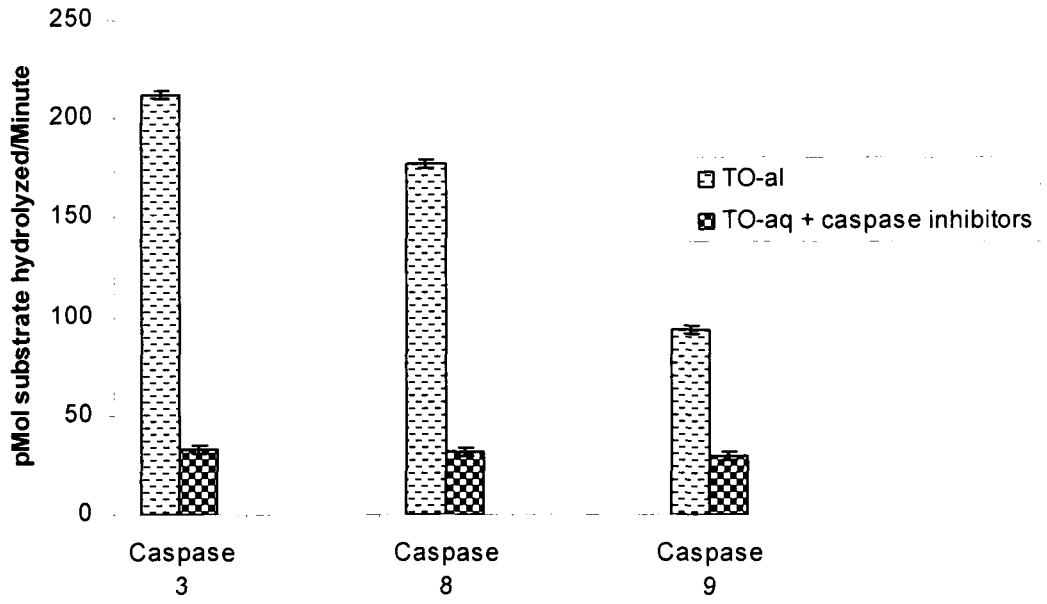

Figure 4.4: Caspase 3,8 and 9 activities of the alcohol extract of *Thuja orientalis* (TO-al).Values are expressed as Mean ± S.E.M.,n=8

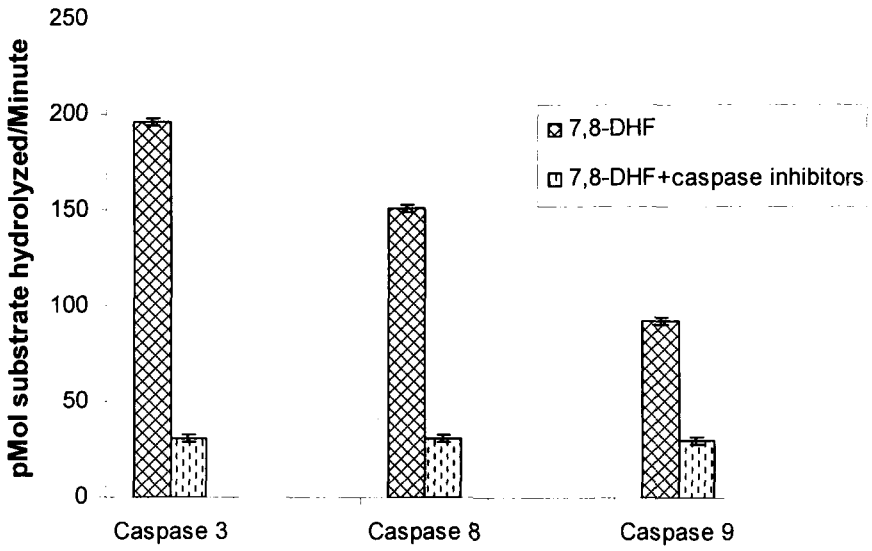

Figure 4.5: Caspase 3,8 and 9 activities of 7,8-dihydroxyflavone (7,8-DHF). Values are expressed as Mean ± S.E.M., n=8

cancer cells with the caspase-3, caspase-8 and caspase-9 inhibitors, DEVD-fmk, z-IEDT-fmk and z-LEHD-fmk respectively followed by test samples for 24 h. As shown in the Figure 4.3, 4.4, 4.5, the presence of caspase inhibitors blocked the effect of test samples (TO-aq, TO-al and 7, 8-dihydroxyflavone) on the viability of cervical cancer cells. Under these conditions, no PARP cleavage was evident (data not shown). Taken together, these results show for the first time that the extracts of *Thuja orientalis* and 7,8-dihydroxyflavone induced apoptosis in cervical cancer cells and the induction of apoptosis was dependent on the caspases mainly caspase-3/CPP32-like proteases as the activity of caspase-3 is the highest. Results further show that the caspase-3 activity of TO-al is the highest and TO-aq is the lowest, the sequence of the caspase-3,8 and 9 activity is TO-al >7,8-dihydroxyflavone >TO-aq. This shows that apoptosis inducing components are concentrated in alcoholic extract. Also the caspase-8 activity is higher than caspase-9, leading to the prediction that extrinsic receptor pathway of apoptosis is involved.

4.3.4. Cytomorphological and histomorphological effects of TO-al on cervical cancer biopsies

Apoptotic cell death is characterized by chromatin condensation, membrane blebbing, intranucleosomal fragmentation of DNA, activation of

caspases, and apoptotic body formation. Cytomorphological investigation for the appearance of carcinoma cells co-cultured along with TO-al (Figure 4.6 B) show better differentiation of nuclear and cellular outlines as well as increased eosinophilia of cytoplasm along with compact and condensed chromatin as compared to cells devoid of any TO-al (Figure 4.6 A) (PAP X 400).Also, TO-al on non-neoplastic ectocervical cytosmears enhanced keratanization process in comparison to control samples devoid of TO-al.

Histomorphological data showed that in comparison to carcinoma cells devoid of any TO-al treatment (Figure 4.7 A), co-culturing with TO-al resulted in augmented eosinophilia of the cytoplasm along with apoptotic body (arrow) having pyknotic nucleus and dense eosinophilic cytoplasm (Figure 4.7 B) (H&E X 400). Interestingly, histomorphological analysis of carcinoma cells co-cultured with 7,8-dihydroxyflavone showed similar results i. e. no apoptotic bodies in untreated cells (Figure 4.8 A), whereas 7,8-dihydroxyflavone-induced augmention in eosinophilia of cytoplasm along with apoptotic body (arrow) having pyknotic nucleus and dense eosinophilic cytoplasm (Figure 4.8 B) (H&E X 400). Thus, the above cytomorphological and histomorphological analysis clearly shows TO-al apoptosis in cervical cancer cells.

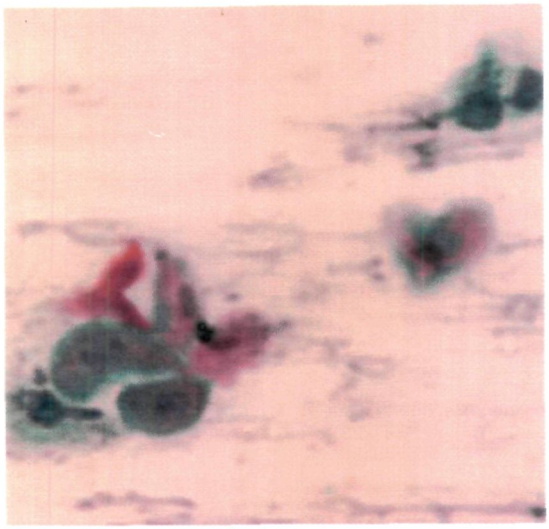

Figure 4.6 A: Cytomorphological results (PAP X 400) showed no apoptotic bodies in control untreated biopsy culture.

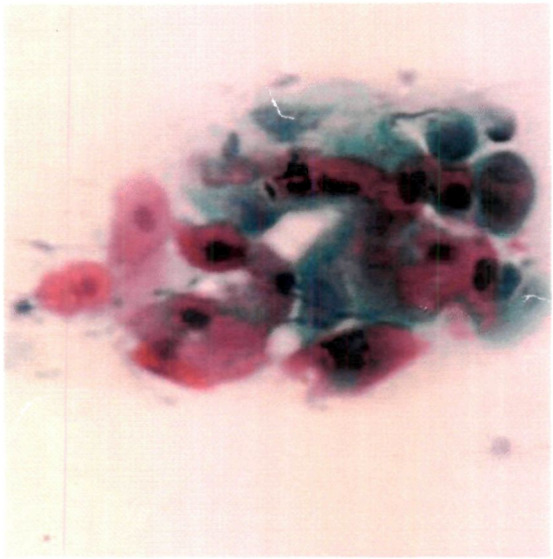

Figure 4.6 B: Cytomorphological results (PAP X 400) of TO-al treated biopsy culture exhibited better differentiation of nuclear and cellular outlines as well as increased eosinophilia of cytoplasm along with compact and condensed chromatin.

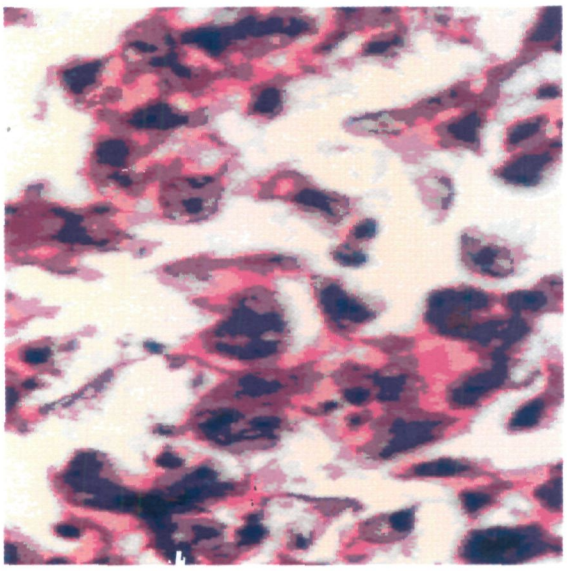

Figure 4.7 A: Histomorphological results (PAP X 400) showed no apoptotic bodies in control untreated biopsy cultures.

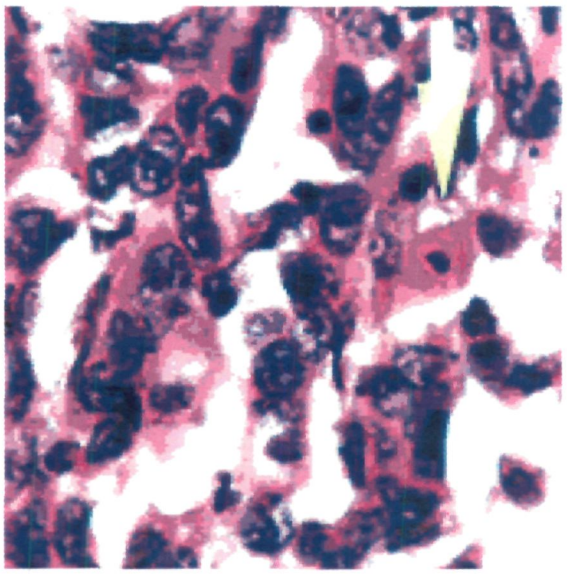

Figure 4.7 B: Histomorphological results (PAP X 400) of TO-al treated samples showed clear apoptosis (see arrow).

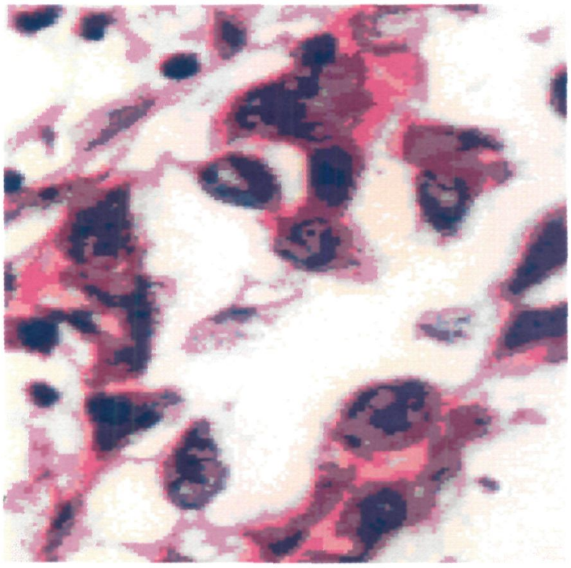

Figure 4.8 A: Histomorphological results (PAP X 400) showed no apoptotic bodies in control untreated biopsy cultures.

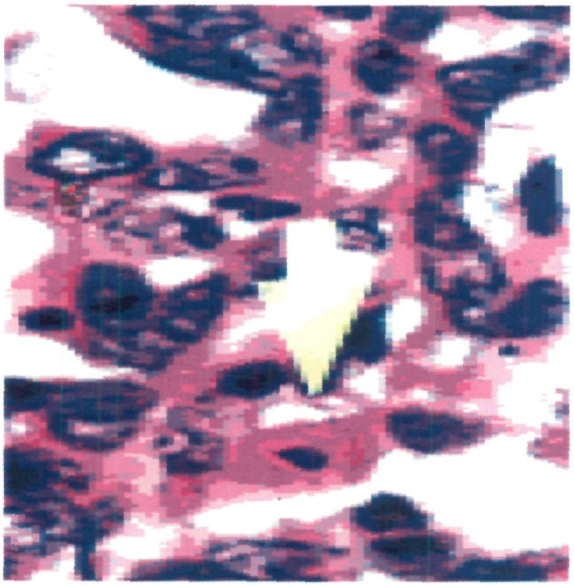

Figure 4.8 B: Histomorphological results (PAP X 400) of 7, 8-dihydroxyflavone treated biopsy cultures showed clear apoptosis.

4.4. DISCUSSION

Apoptosis is one of the most investigated areas in carcinogenesis because of therapeutic interventions (Raff, 1998; Gastman, 2001). It is a highly regulated cell death program that is induced in cells as a suicide response due either to unfavourable growth conditions or exposure to pro-apoptotic external stimuli (Petit et al., 1995).Caspases are recognized as key molecules in apoptotic response. Apoptosis occurs via two major different activation pathways (Green , 1998; Ashkenazi and Dixit, 1998). One pathway involves changes in mitochondrial transmembrane potential, leading to the release of cytochrome-c, which then binds the apoptosis activating factor 1 and procaspase-9, resulting in the activation of caspase-9 by proteolytic cleavage. The other pathway starts with death receptor ligation or Fas/FasL interaction, followed by oligomerization of the receptor, recruitment of Fas-associated death domain protein (FADD), and activation of caspase-8 (Stennicke and Salvesan, 1998). Both caspase-9 and caspase-8 are defined as initiator caspases and can in turn activate caspase-3, the executor of apoptosis (Stennicke and Salvesen, 1998; Zha et al., 2000). Also, cross communication exists between the two pathways, as caspase-8 may activate caspase-9 via Bid, a member of bcl-2 family (Islam et al., 2000).

Extensive studies are available on the mechanism of chemopreventive effect of natural antioxidants on cancerous cell lines (Islam et al., 2000, Singh et al., 2002). In some cell lines they have shown to induce apoptosis, whereas in some cell lines they have shown adverse effects. Thus, in search of a potent natural agent / antioxidant, that may induce apoptosis in cervical cancer biopsies, the present study involves utilization of the leaf extracts of *Thuja orientalis*. Thus, we probed the mechanism of *Thuja orientalis* mediated apoptosis in monocytes of cervical cancer patients. Our results showed involvement of activation of caspase-3. Alcohol extract of *Thuja orientalis* (TO-al) was highly effective in inhibiting the viability of cervical cancer monocytes by PARP cleavage in response to activation of caspase-3. This resulted in the loss of normal PARP function, which irreversibly commits the cell to die (Islam et al., 2000; Singh et al., 2002; Islam et al., 2002; Orth et al., 1996).

In the present study, we report that the activities of caspase-3, caspase-8 and caspase-9 in cervical cancer monocytes were activated by the extracts of *Thuja orientalis*, indicating that both death receptor-related apoptotic pathway and the mitochondria-related pathway were activated. On the contrary, suppression or reduction in cell viability was inhibited by

inhibitors of caspase-3, 8 and 9 namely Z-DEVD-FMK, Z-IETD-FMK and Z-LEHD-FMK respectively, which specifically blocked PARP cleavage.

Also, apoptosis occured in cervical cancer biopsies cultured with the alcohol extract of *Thuja orientalis* (TO-al) as shown by the cytomorphological and histopathological studies (Figure 4.6 A, Figure 4.6 B, Figure 4.7 A, Figure 4.7 B). Histopathological results of TO-al treated cells were compared with 7, 8- dihydroxyflavone (standard taken) treated cells (Figure 4.8 A and Figure 4.8 B) and were found comparable.

In conclusion, the alcohol extract of *Thuja orientalis*, is a potent inducer of apoptosis in biopsies of cervical cancer patients, and that, induction of TO-al mediated apoptosis involved activation of caspase-3/CPP32, caspase-8 and caspase-9, particularly caspase-3, a member of the caspase family of 13 aspartate-specific cysteine proteases that play a central role in the execution of the apoptotic program (Alnemri et al., 1996; Cohen et al., 1997).

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CHAPTER-5

ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC CONTENT OF FIVE DIFFERENT SOLVENT EXTRACTS OF THE FRUIT OF FICUS RACEMOSA

5.1. INTRODUCTION

Ficus racemosa Linn synonymn *Ficus glomerata* is a large, evergreen tree, 40 to 50 ft in height and common through all parts of India.

Biological activity: In Ayurvedic system of medicine almost every part of this plant is used for medicinal purposes (Chopra et al., 1956; Singhal and Baslas, 1978, Majumdar and Momin, 1960). The bark, leaves and unripe fruit are astringent, carminative, stomachic and vermicide. The roots are used as a medicine against hydrophobia. The fruit of *Ficus racemosa* is edible and efficacious in diabetes (Gupta, 1964; Patil et al., 2006). It is also effective against leprosy, diseases of the blood, fatigue, bleeding nose and cough (Trivedi et al., 1969). Its bark is helpful against asthma and its leaves are used against bronchitis. The plant is used locally to relieve inflammation of skin wounds, sprains and fibrosis. The alcoholic extract of the stem bark of the plant possessed antiprotozoal activity against *Entamoeba histolytica.* It is used in the treatment of mumps, smallpox, haematuria & inflammatory conditions (Mandal et al., 2000) .The leaf extract of *Ficus racemosa* showed protection against liver damage caused by carbon tetrachloride in rats (Mandal et al., 1999).It is also reported to have antibacterial activity (Mandal et al., 2000a). The chemomodulatory effect of *Ficus racemosa*

against ferric nitrilotriacetate (Fe- NTA) induced renal carcinogenesis was reported recently (Khan and Sultana, 2005). Li et al. (2004) have reported anti-inflammatory glucoside from *Ficus racemosa*. The bark extract of Ficus *racemosa* shows anti-pyretic activity (Rao et al., 2002), glucose lowering efficacy (Rao et al., 2002a) and antitussive potential (Rao et al., 2003).

Earlier studies have shown significant anti-inflammatory, analgesic and antipyretic activity of aqueous, alcohol and petroleum ether extracts in rodents from the leaves.

Chemistry: Phytochemical studies of *F.racemosa* revealed the presence of several compounds including lupeol (Singhal and Saharia, 1980), β- sitosterol (Sen and Chaudhury, 1971; Bhatt and Agarwal, 1973; Chandra et al., 1979; Singhal and Saharia, 1980) and stigmasterol (Singhal and Saharia, 1980). This plant has also been reported to contain tannins, kaempferol, rutin, arabinose, bergapten, psoralenes, flavonoids, ficusin, coumarin and phenolic glycosides (Baruah and Gohain, 1992). Psoralen, an antifungal compound has been isolated from the extract of the leaves of *Ficus racemosa* using 50% methylene chloride in hexane flash column (Deraniyagala et al., 1998).

Most of the biological studies on *Ficus racemosa* are done on its bark and leaves. The edible fruits have not been studied so far. The present study

aims to study the antioxidant potential of the edible fruits of this plant. Five different solvent have been used to prepare the fruit extracts.

5.2. MATERIALS AND METHODS

5.2.1. Plant material

Ripe fruits of *Ficus racemosa* were handpicked, washed and dried in air at room temperature. Dried fruits were grinded to obtain a powdered material. This powdered material (160 g) was extracted with various solvents.

5.2.2. Preparation of the fruit extract

Powdered fruit material (160 g) was repeatedly extracted in a 1000 ml round bottomed flask with 500 ml solvents of increasing polarity starting with petroleum ether, chloroform, ethyl acetate, acetone and methanol. The reflux time for each solvent was four hours. The extracts were cooled at room temperature, filtered and evaporated to dryness under reduced pressure in a rotatory evaporator (Buchi rotavapor). The yield is given in Table 5.1.

5.2.3. Determination of total phenolic content

Total phenolic content was determined by Folin-Ciocalteau Method. It is described in detail in Chapter 2.

5.2.4. Antioxidant activity measurement

Antioxidant activity was measured by determining the reducing power, DPPH radical scavenging, superoxide anion radical scavenging, inhibitory effect on Fenton reaction induced oxidative DNA damage and inhibition of β-carotene bleaching. The procedures are described in detail in Chapter 2.

5.3. RESULTS

5.3.1. Yield of various solvent extracts of *Ficus racemosa*

The highest yield of extract is obtained in methanol followed by petrol, chloroform, ethyl acetate and acetone (Table 5.1). There is no correlation between the yield and antioxidant activity.

Table 5.1: Percent yield of various solvent extracts of the fruit of *Ficus racemosa*

Extract	Yield (%)
Petroleum ether	3.316
Chloroform	2.84
Acetone	1.29
Ethyl acetate	2.153
Methanol	6.443

5.3.2. Total phenolic content

As shown in Table 5.2, methanol extract has highest total phenolic content as expressed in terms of gallic acid equivalents that is 244.09 GAE (in μg/100 μg of dry extract). The order of decreasing total phenolic content is methanol extract (F-Me)> ethyl acetate extract (F-EA) > acetone extract (F-Ac) > chloroform extract (F-Chl) > petrol extract (F-Pet). Thus, highest amount of phenolic compound are extracted in methanol and lowest in petrol.

Table 5.2: Total phenolic content of the fruit extracts of *Ficus racemosa*. Values are expressed as gallic acid equivalents (μg/100 μg of dry weight).

Extracts (100 μg)	Absorbance (760 nm)	Gallic acid equivalents (μg/100 μg of dry weight)
F-Me	0.271	244.09
F-EA	0.164	146.82
F-Ac	0.138	123.18
F-Chl	0.092	81.36
F-Pet	0.087	76.82

5.3.3. Reducing power of the fruit extracts of *Ficus racemosa*

The reducing powers determined by the present assay depend on the redox potentials of the compounds present in the fruit extracts of *Ficus racemosa*. Reducing power of different solvent extracts of *Ficus racemosa* is shown in Figure 5.1. Methanol extract is showing highest reducing power followed by ethyl acetate extract. The order of the reducing power is methanol extract > ethyl acetate extract > acetone extract > chloroform extract > petrol extract.

5.3.4. DPPH radical scavenging activity

As shown in Figure 5.2, DPPH radical scavenging activity of the methanol extract is remarkably higher than that of other solvent extracts. At a concentration of 150 μg/ml, methanol extract is able to scavenge 52.16% of DPPH free radical, while at the same concentration ethyl acetate, acetone, chloroform and petrol extract showed 23.68%, 19.24%, 15.30% and 14.26% radical scavenging activity, respectively. The % radical scavenging activity of the fruit extracts showed dose dependency and increased with the increasing concentration of the extracts. At a concentration of 500 μg/ml, the free radical scavenging activity of methanol extract is above 90% that is 91.48 %, ethyl acetate (67.53%) and acetone (52.9%) extracts also scavenged above 50% of DPPH radical at the same concentration while the

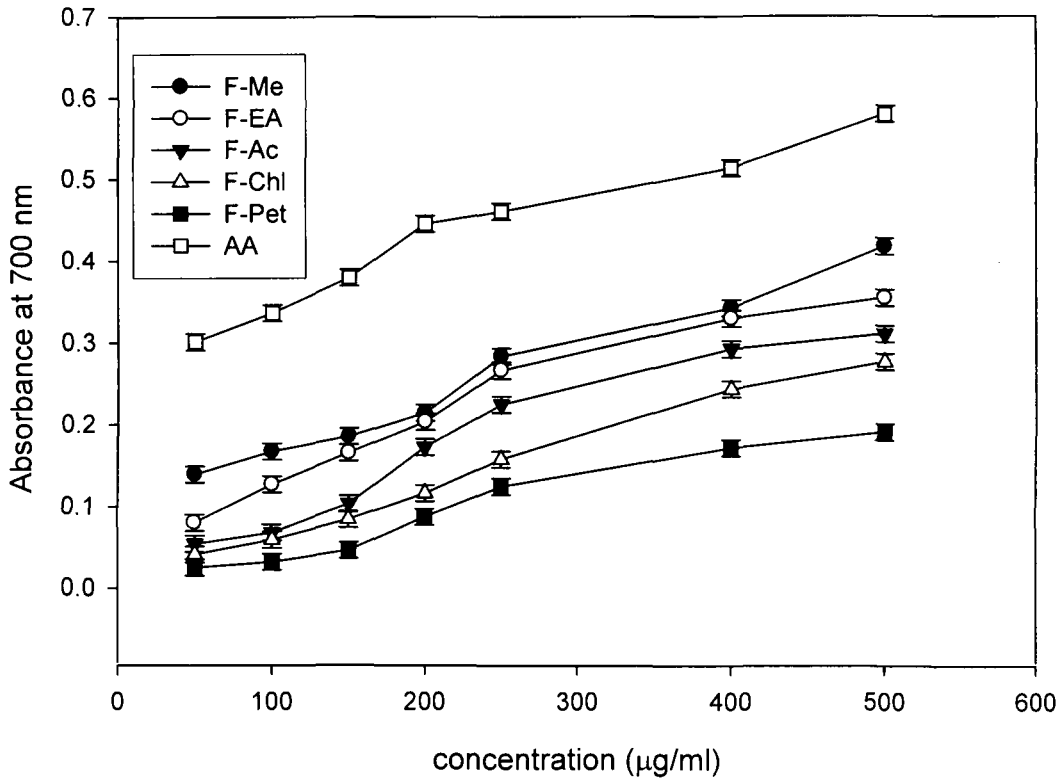

Figure 5.1: Reducing power of the fruit extracts of *Ficus racemosa*.Ascorbic acid is used as a standard.Values are expressed as Mean ± S.E.M., n=3. F-Me=methanol extract;F-EA=ethyl extract; F-Ac=acetone extract; F-Chl= chloroform extract; F-Pet=petrol extract;AA=ascorbic acid.

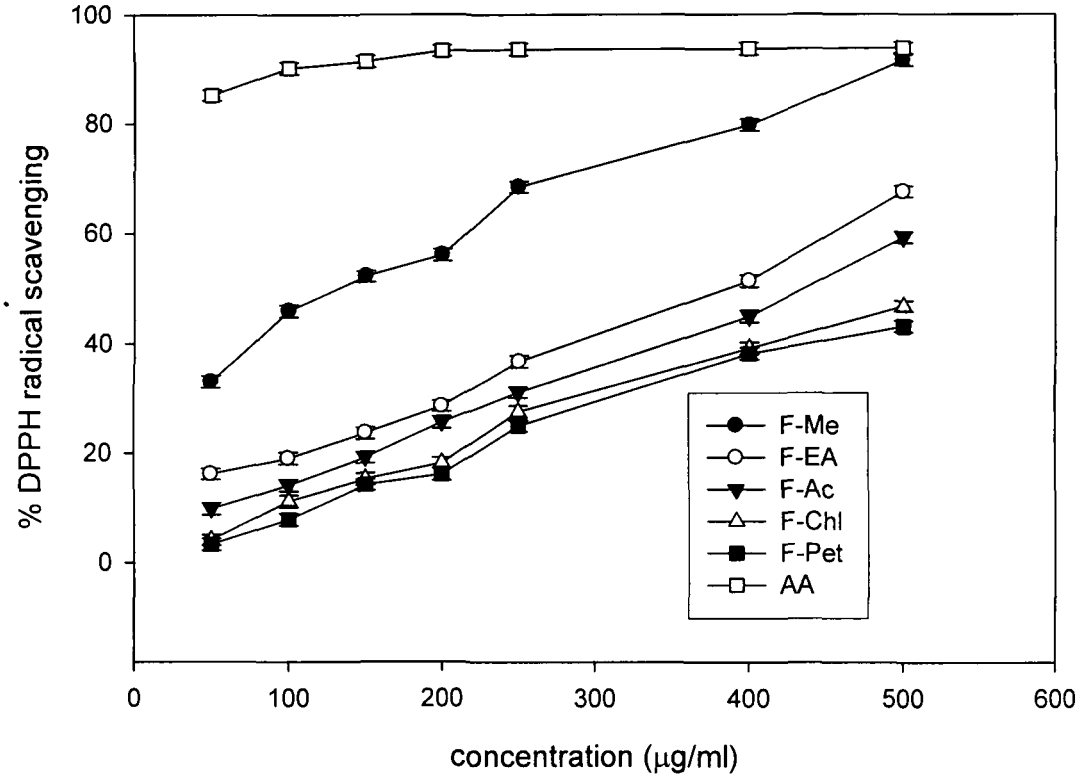

Figure 5.2 : DPPH radical scavenging activity of the fruit extracts of *Ficus racemosa*. Ascorbic acid is used as standard. Values are expressed as Mean ± S.E.M., n=3.F-Me=methanol extract;F-EA=ethyl acetate extract; F-Ac=acetone extract; F-Chl=chloroform extract;F-Pet=petrol extract; AA=ascorbic acid.

radical scavenging activity of chloroform and petrol extract is less than 50% at 500 μg/ml.

5.3.5. Superoxide radical scavenging activity

As shown in Figure 5.3, the superoxide radical scavenging activity of the methanol extract is highest .There is dose dependent response and a linear increase with the increasing concentration of extracts. At a concentration of 200 μg/ml, methanol, ethyl acetate and acetone extracts scavenges more than 50% of superoxide anion radical while choloroform and petrol extract scavenges less than 50% of superoxide anion radical. Methanol extract (F-Me) showed highest scavenging capacity and scavenges 79.12 % of superoxide anion radical at a concentration of 200 μg/ml while ethyl acetate (F-EA), acetone (F-Ac) ,chloroform (F-Chl) and petrol extract (F-Pet) showed 64.20%, 60.54%, 40.12% and 32.70% superoxide anion radical scavenging ability, respectively at 200 μg/ml.

5.3.6. Inhibition of Fe (II) - EDTA- H_2O_2 induced oxidative DNA damage

The ability of the fruit extracts of *Ficus racemosa* to modulate Fenton reaction induced oxidative DNA damage is studied by this assay. Control experiments (not shown) established that heat denatured DNA underwent 100 % hydrolysis following treatment with S_1 nuclease, whereas native DNA

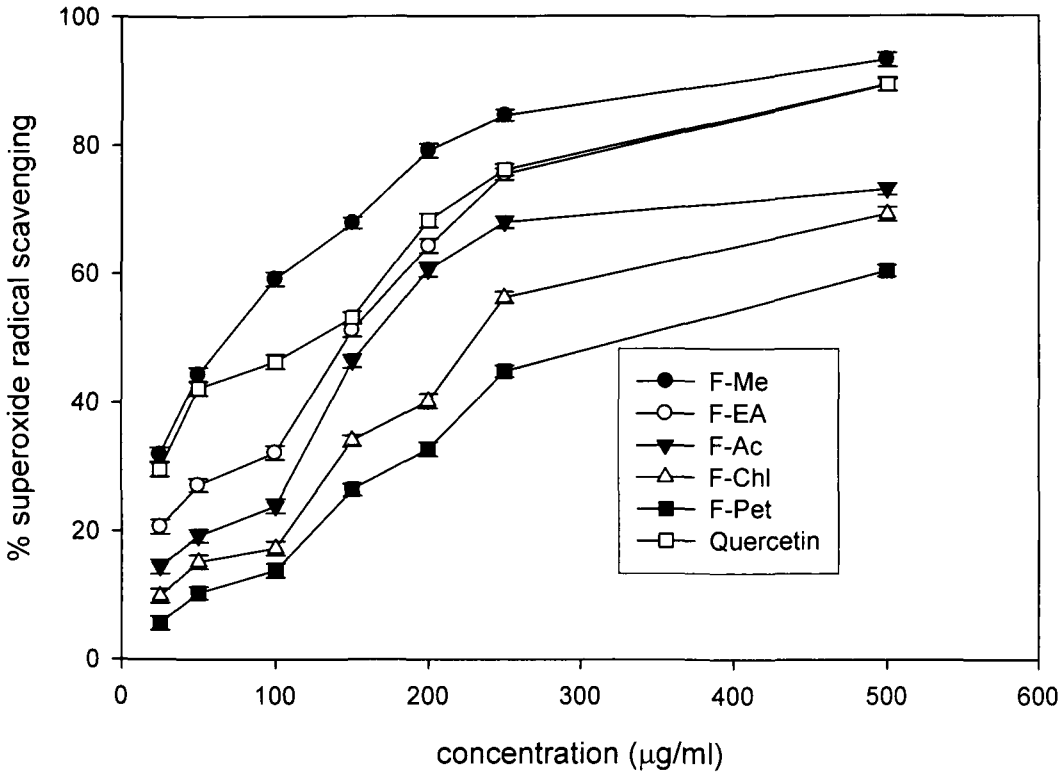

Figure 5.3: Superoxide radical scavenging activity of the fruit extracts of *Ficus racemosa*.Quercetin is used as a standard. Values are expressed as Mean ± S.E.M., n=3.F -Me=methanol extract;F-EA=ethyl acetate; F-Ac=acetone extract;F-Chl=choloroform extract;F-Pet=petrol extract

resulted in < 10% conversion. S_1 nuclease hydrolysis of DNA decreases in a dose dependent manner with the increasing concentration of the fruit extracts (Figure 5.4), thereby showing that the fruit extracts are inhibiting DNA damage induced by Fenton reaction. Fenton reaction produces hydroxyl radical which is a highly reactive free radical causing damage to DNA, proteins and other biomolecules. Antioxidants are capable of neutralizing hydroxyl radical by donating an electron or hydrogen atom, thus in this assay fruit extracts of *Ficus racemosa* are showing good antioxidant activity. At a concentration of only 100 μg/ml, methanol extract of the fruits of *Ficus racemosa* (F-Me) inhibited DNA hydrolysis by 80.12 %. Ethyl acetate extract showed 69.04% inhibition at the same concentration while acetone, chloroform and petrol extract showed 54.10%, 47.62% and 41.03% inhibition of DNA hydrolysis, respectively (Figure 5.5). A standard antioxidant quercetin was used for comparison with the fruit extracts. Quercetin showed 76.34% inhibition of DNA hydrolysis at a concentration of 100 μg/ml while methanol extract (F-Me) showed 80.12% inhibition of DNA hydrolysis at the same concentration. Thus, methanol extract is showing higher inhibitory effect on DNA hydrolysis compared to quercetin.

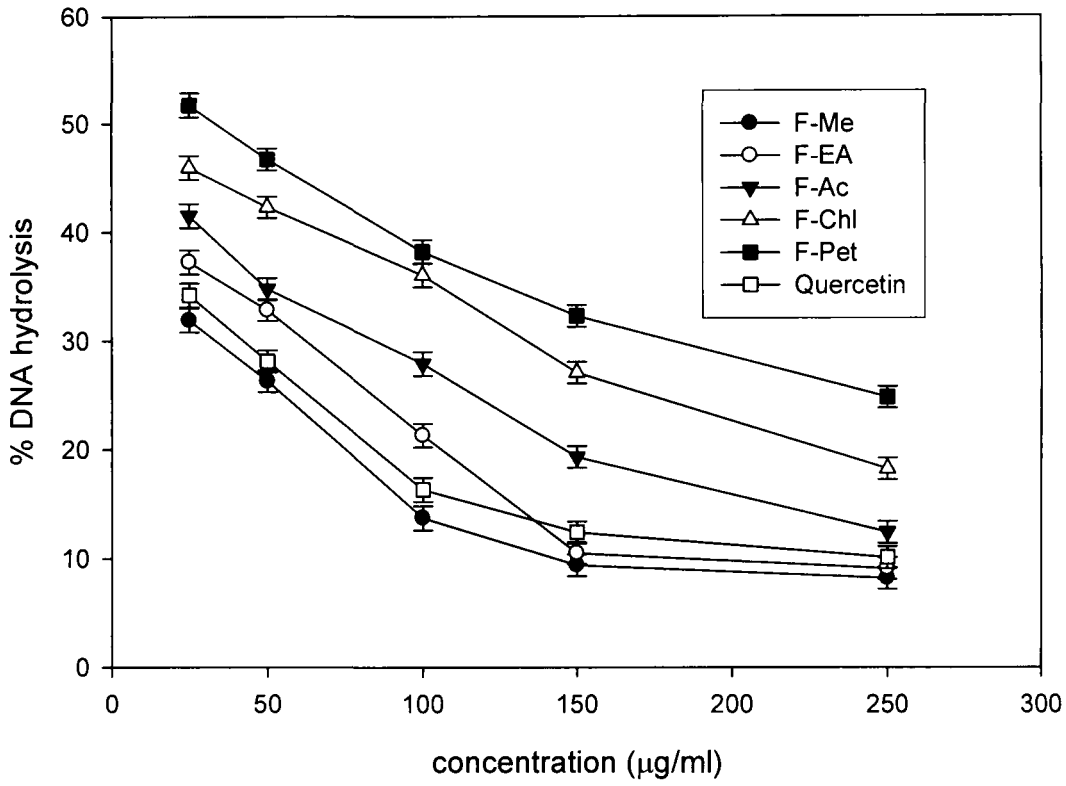

Figure 5.4: Decrease in degradation of DNA in the presence of the fruit extracts of *Ficus racemosa*. Quercetin is used as a standard. Values are expressed as Mean ± S.E.M., n=3.F-Me =methanol extract; F-EA =ethyl acetate extract; F-Ac=acetone extract; F-Chl= chloroform extract; F-Pet=petrol extract.

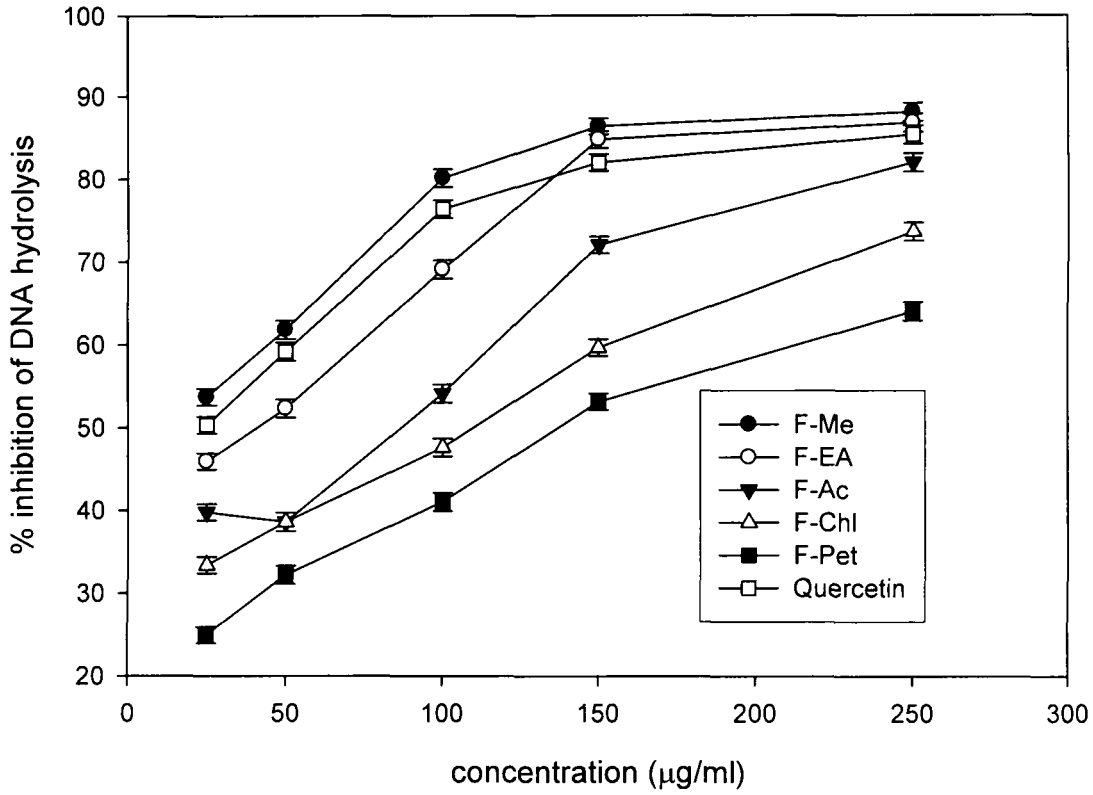

Figure 5.5: Inhibition of DNA hydrolysis by the fruit extracts of *Ficus racemosa*. Quercetin is used as a standard . Values are expressed as Mean ± S.E.M., n=3.F-Met=methanol extract;F-EA=ethyl acetate extract; F-Ac=acetone extract;F-Chl=chloroform acetate;F-Pet=petrol extract.

5.3.7. Antioxidant Activity in β-carotene-linoleate system

The antioxidant activity of the fruit extracts of *Ficus racemosa* as measured by the bleaching of β-carotene is presented in Figure 5.6. It can be seen that *F. racemosa* fruit extracts prepared by different solvents exhibited various degrees of antioxidant activity. Methanol extract (F-Me) shows the highest activity followed by ethyl acetate and acetone extract. At a concentration of 100 μg/ml, methanol, ethyl acetate, acetone, chloroform and petrol extract inhibited the bleaching of β-carotene by 56.89, 37.82, 30.59, 22.16 and 20.88, respectively.

5.4. DISCUSSION

Fruits and vegetables are the main sources of antioxidant vitamins (vitamin E, vitamin C, precursor of vitamin A i.e., β- carotene), which act as free radical scavengers, making these foods essential to human health (Elliott, 1999). However, more than 80% of the total antioxidant capacity in fruits and vegetables come from ingredients other than antioxidant vitamins, indicating the presence of other potentially important antioxidants in these foods (Miller and Rice-Evans, 1997) and phenolic compounds are the dominant antioxidants that exhibit scavenging activity on free radicals (Namiki, 1990).

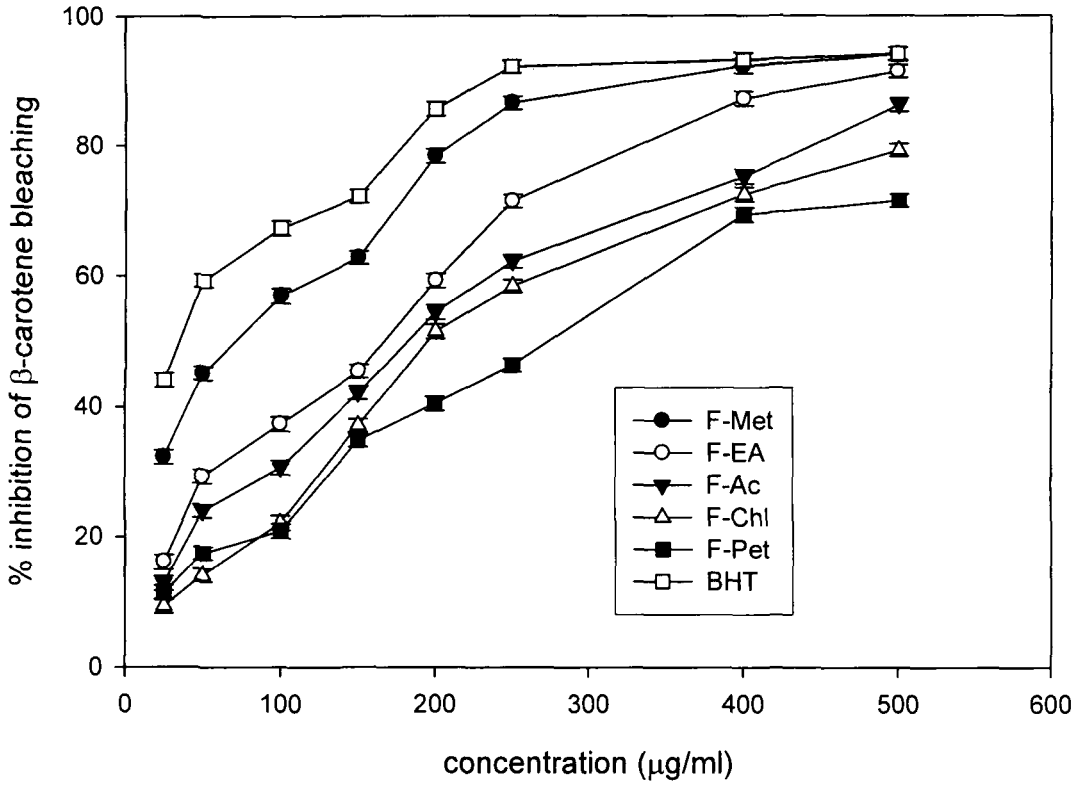

Figure 5.6: Antioxidant activity of the fruit extracts of *Ficus racemosa* as determined by β-carotene-linoleate model.BHT is used as a standard. Values are expressed as Mean ± S.E.M., n=3.F-Met=methanol extract; F-EA=ethyl extract;F-Ac=acetone extract; F-Chl=chloroform extract; F-Pet=petrol extract.

In our study, the decreasing order of antioxidant activity among the *F. racemosa* fruit extracts assayed through all the five methods was found to be methanol extract (F-Me)> ethyl acetate extract (F-EA)> acetone extract (F-Ac)> chloroform extract (F-Chl)>petrol extract (F-Pet). This order is similar to the total phenolic content of the extracts. Thus high total phenolic content of methanol extract (244.09 GAE) appears to be responsible for its highest antioxidant activity as compared to other solvent extracts of the fruit. Several reports have conclusively shown close relationship between total phenolic content and antioxidative activity of the fruits and vegetables (Deighton et al., 2000; Vinson et al., 1998). Further, it has been reported that *Ficus racemosa* contain tannins, kaempferol, rutin, flavonoids and phenolic glycosides (Baruah and Gohain, 1992), thus the antioxidant activity of the fruit extracts could be attributed to the presence of these compounds.

Figure 5.1 shows the reducing power of the extracts of *F.racemosa* and standard L-ascorbic acid using the potassium ferricyanide reduction method. The reducing power of the extracts increased with increasing concentration. Methanol extract showed stronger reducing power than other solvent extracts of the fruit of *Ficus racemosa*. Figure 5.2 illustrated the DPPH radical scavenging activity of the fruit extracts of *Ficus racemosa*. DPPH is a stable free radical which is reduced in the presence of a hydrogen

donating antioxidants. The radical scavenging activity of methanol extract is considerably higher than other solvent extracts, at 150 µg/ml DPPH radical scavenging of methanol extract is 52.16% while ethyl acetate extract scavenges only 23.68% at the same concentration. Phenolic compounds are hydrogen donating antioxidants, thus higher radical scavenging activity of methanol extract (F-Me) may be attributed to higher amount of hydrogen donating phenolic antioxidants in methanol extract.

Superoxide radical is known to be a very harmful species to cellular components as a precursor of more reactive oxygen species (Cheeseman and Slater, 1993). The fruit extracts of *Ficus racemosa* are found to be an efficient scavenger of superoxide radical (Figure 5.3) generated in xanthine-xanthine oxidase system. Methanol extract (F-Me) is showing highest superoxide anion scavenging activity, scavenging 79.12 % and 93.1 % superoxide radical at a concentration of 250 µg/ml and 500 µg/ml, respectively. It is showing higher activity as compared to the standard antioxidant quercetin.

Protective effects of the fruit extracts of *Ficus racemosa* on DNA oxidative damage was studied using Fenton reaction to induce DNA damage followed by S_1 nuclease hydrolysis. The oxidative stress due to oxygen and various radical species is associated with the induction of DNA single and

double strand breaks and is considered to be the first step in several human degenerative diseases, such as, cancer and ageing (Imlay et al., 1988; Meneghini, 1997; Arouma, 1998). In the Fe (II)-EDTA-H_2O_2 induced oxidative DNA damage assay, all the fruit extracts of *Ficus racemosa* showed inhibitory effect on the DNA hydrolysis and subsequent oxidative damage. S_1 nuclease hydrolysis of DNA decreases in a dose dependent with the increasing concentration of the fruit extracts (Figure 5.4). As shown in results (Figure 5.5), methanol extract showed highest inhibitory effect on DNA hydrolysis, at a concentration of 100µg/ml, methanol extract of *F. racemosa* fruits (F-Me) inhibited hydrolysis of DNA by 80.12%, while quercetin, a standard antioxidant showed 76.34% inhibition at the same concentration. As compared to quercetin, only methanol extract is showing higher inhibitory potential,while other solvent extracts of *F.racemosa* fruits showed less inhibitory activity as compared to quercetin but all the fruit extracts showed good inhibition potential ranging from 80.12% to 41.03% at a concentration of 100µg/ml.

The percent (%) inhibition of β-carotene bleaching is summarized in Figure 5.6. In this assay also, methanol extract showed highest inhibiting potential against β-carotene oxidation but its activity was lower than BHT, which shows 67.51% inhibition of β-carotene bleaching at a concentration of

100 µg/ml as compared to methanol extract which inhibited 56.89% bleaching at 100 µg/ml.

In conclusion, the results clearly indicate that the extraction with methanol not only gives high yield of the extract and total phenols , but the methanol extract also shows high antioxidant activity, which was confirmed by various methods used for estimation of antioxidant activity. Thus, selective extraction of antioxidant from natural sources by appropriate solvent is very important in obtaining fractions with high antioxidant activity. Also, the fruit extracts of *Ficus racemosa* have got good antioxidant activity and the consumption of the fruit of *Ficus racemosa* is going to be beneficial in preventing oxidative stress related degenerative diseases and could slow the process of ageing besides being beneficial in diabetes.

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CHAPTER-6

ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC CONTENT OF SOME MEDICINAL HERBS

6.1. INTRODUCTION

Medicinal herbs possess many phytochemicals which are efficient scavengers of free radicals. The present study was conducted to assess the antioxidant activity of some locally collected medicinal herbs selected from the family Euphorbiaceae. Asteraceae, Scrophuliaraceae and Solanaceae. Two herbs were selected from each family. The list is given below:

Family	Herbs Studied
Asteraceae	*Pluchea lanceolata* *Vernonia cinerea*
Euphorbiaceae	*Acalypha indica* *Phyllanthus fraternus*
Scrophuliaracea	*Lindenbergia indica* *Scoparia dulcis*
Solanaceae	*Solanum nigrum* *Withiania somnifera*

Herbs studied:

Family Asteraceae

Pluchea lanceolata

Biological activity: *Pluchea lanceolata* C.B.Clarke (Family Asteraceae) commonly known as "Rasana" is well known medicinal herb

among the herb collectors and traders of Chattisgarh. All parts of "Rasana" possess valuable medicinal properties but its leaves are used most commonly as medicine (Chawla et al., 1990). The plant is used for the treatment of rheumatoid arthritis (The Wealth of India, 1969). *Pluchea lanceolata* suppressed Ferric nitrilotriacetate (Fe-NTA) induced renal carcinogenesis (Jahangir and Sultana, 2006), and has anti-inflammatory activity (Kaith and Kaur, 1991).

Chemistry: Flavonoids (The Wealth of India, 1969), pluchine (Dasgupta et al., 1967), pentacyclic triterpenes (Chawla et al., 1991; Alam et al., 1994), sterols (Chawla et al., 1991; Alam et al., 1994a), and aliphatic constituents (Chawla et al., 1990, 1991, 1992; Alam et al., 1994, 1994a), have been reported from the aerial part of the plant.

Vernonia cinerea

Vernonia cinerea (Family: Asteraceae) is a weed common throughout India and it is well known as "Sahadevi" (Sanskrit), Naichette (or) Mukuthipundu (Nadkarni, 1976).

Biological activity: In traditional system of medicine the whole plant with its small flowers is used medicinally to promote perspiration in febrile conditions (Kirthikar and Basu, 1975). The flower extract of the plant is used in the treatment of adjuvant-induced arthritis (Latha et al., 1998). An

aqueous ethanolic (50%) extract of the plant showed activity against Rani Khet viral disease. Fresh juice of leaves is locally applied for the extraction of guinea worms. The seeds are also used as an antihelminthic and alexipharmic, and they are quite effective against round worms and thread worms. They are also given for coughs, flatulence, intestinal colic and chronic skin diseases. A paste of seeds with lime juice is used to destroy pediculi. The flowers are used to treat conjunctivitis, fever, and rheumatism. Phenolic constituents of *V. cinerea* inhibited chemiluminscence generated by zymosan activated polymorphonuclear leucocytes (Vanden Berg and Sluis, 1999).

Chemistry: β-Amyrin, lupeol and their acetates, β-sitosterol, stigmasterol, α-sitosterol, stigmasterol, α-spinasterol and phenolic resin have been isolated from the whole plant. Misra et al. (1984, 1984a, 1984b) isolated several terpenoids, a natural sterol and an aliphatic acid from *Vernonia cinerea.*

Family Euphorbiaceae

Acalypha indica

Acalypha indica Linn. (local name muktajhuri, muktajari, murkanta) is a small herb belonging to the family Euphorbiaceae. It generally occurs as

a weed in gardens in India, Bangladesh, tropical Africa and Phillipines. It is about 30-75 cm in height with numerous branches.

Biological activity: Different parts of the plants are used traditionally for the treatment of diseases, fruits are used in asthma, cough, bronchitis and earache. Fresh juice of leaves is applied with oil, salt or lime in rheumatoid arthritis and to cure scabies and other skin infections, the powdered leaves are used for bedsores and maggot infested wounds.

Alcohol extracts of the tender shoots, leaves and roots showed activity against *Micrococcus pyogens* var, *aureus* and *Eschrechia coli*. Aqueous extracts of *Acalypha indica* showed antimicrobial and wound healing activities (Reddy et al., 2002). Plant juices extracted from *Acalypha indica* are mixed with duck feed, according to the locals the plant juices are rich in calcium and thus increased egg production. The leaf extract of *Acalypha indica* showed larvicidal activity against malaria vector (Govindarajan et al., 2008), anti-inflammatory activity (Gnanasekar et al., 2003) and antifertility activity (Hiremath et al., 1999).

Chemistry: The plant contains a cyanogenic glucoside and two alkaloids viz. acalyphine and triacetonamine, possibly a degradation product of the glucoside. The other constituents are n-octasosanol, β- sitosterol,

kaempferol, quebrachitol, tannin, resin and an essential oil (Nahrstedt et al., 2006).

Phyllanthus fraternus

Phyllanthus fraternus (Euphorbiaceae) is an annual herb, widely distributed in the northern region of India, Pakistan and introduced into Saudi Arabia, Africa and West Indies (Abedin et al., 2001). It is used as a folklore remedy for the treatment of various diseases of liver by traditional healers and tribals (Kirtikar and Basu, 1975).

Biological activity: *Phyllanthus fraternus* showed protective effect against alcohol (Sebastian and Shetty, 1999) and allyl alcohol (Sailaja et al., 2006) induced oxidative stress in liver mitochondria. Administration of the aqueous extract of the *Phyllanthus fraternus* significantly decreased the thioacetamide (Padma and Setty, 1997) or carbon tetrachloride (Padma and Setty, 1999) induced lipid peroxidation *in vivo* and protected the liver from the hepatotoxin induced toxicity. Petrol extract of whole plant (Bhatnagar et al., 1961) and the leaf extract (Bhowmick and Chaudhari, 1982) of *Phyllanthus fraternus* were reported to have antifungal activity. Ethanol extract of whole plant showed anticancer activity in the mouse and antispasmodic activity on isolated guinea pig ileum (Dhar et al., 1968). The aqueous extract from its leaves is reported to have hypoglycemic effect in

normal as well as alloxan diabetes rabbits (Ramakrishnan et al., 1982). The hydroalcoholic extract given intraperitoneally, produced significant inhibition of acetic acid induced abdominal constrictions (Santos et al., 2000). Ahmed et al. (2002) have reported that the methanolic fraction was the most active among all fractions studied in protecting liver against carbon tetrachloride induced toxicity.

Family Scrophulariaceae

Lindenbergia indica

Lindenbergia indica (L.) (Scrophulariaceae) is an erect annual herb, 10-30 cm in height, found throughout India. It is commonly found in crevices on old walls and banks of ancient monuments. The plant possesses a faint aromatic odour and is slightly bitter.

Biological activity: The juice of the plant is given in chronic bronchitis and mixed with that of coriander applied to skin eruptions (Chopra et al., 1956).

Chemistry: Phytochemical studies of *Lindenbergia indica* reveal the presence of saponins (Tiwari and Choudhary, 1979), oleanic acid, 7-hydroxyflavone and quercitrin (Tiwari and Choudhary, 1980).

Scoparia dulcis

Scoparia dulcis, common name: Vassourinha is an erect annual herb growing upto a half-meter in height and is widely distributed in many tropical countries.

Biological activity: The antitumorous activity of Scopadulcic acid B, one of Vassourinha's main active constituents was demonstrated in a 1993 study (Nishino et al., 1993). Scopadulcic acid B and Scopadulin demonstrated antiviral properties in two prior studies, including against Herpes Simplex I *in vivo* in hamsters (Hayashi, T. et al., 1990; Hayashi, K. et al., 1988). An ethanol extract of the whole plant of *S. dulcis* demonstrated an ability to inhibit receptor binding to both dopamine and serotonin receptors (Hasrat et al., 1997). *Scoparia dulcis* also showed anti-inflammatory (Ahmad et al., 2001), antidiabetic (Latha and Pari, 2003, 2004, 2004a, 2004b, Pari and Latha, 2004, 2004a), antiulcer (Babincova et al., 2008), antimalarial (Riel et al., 2002) and anticancer (Ahsan et al., 2003) activities.

Chemistry: Phytochemical screening of Vassourinha has shown that it is a source of novel flavonoids (Hayashi, T. et al., 1988) and terpene (Hayashi, T. et al., 1987, 1990, 1990a, 1992, Mahato, et al., 1981). Many of Vassourinha's tested biological activities are attributed to these

phytochemicals; the main ones being scopadulcic acids A & B (Hayashi,T. et al., 1990a, 1992), scopadiol, scopadulciol, scopadulin, scoparic acids A,B,C (Ahmed and Jakupovic, 1990; Hayashi, T. et al., 1987, 1990, 1992; 1993).

Family Solanaceae

Solanum nigrum

Solanum nigrum L. (Solanaceae) is an herbal plant indigenous to Asia and grows wildly and abundantly in open fields. It has been used in traditional Oriental medicines for treating various kinds of tumors and is believed to have various biological activities (Son et al., 2003).

It produces black coloured fruits, fruits of some strains are eaten in pies and are also used for making preserves. Young shoots and leaves are cooked as a potherb. The black berries and whole plant are used for food in Java.

Biological activity: The juice prepared in various manners, is said to be a folk remedy for tumors and cancer, as in the case with the leaf. A poultice of the root is said to remedy scirrhous tumors of the rectum. Medicinally many parts of the plant are used; berries are considered diuretic and are used for eye diseases, fevers, and hydrophobia; juice of the plant is considered cathartic, diuretic, hydrogogue, laxative, alternative emollient

and is used for chronic enlargement of the liver, in blood-spitting, piles, and dysentery.

Previous investigations have shown that extracts of *Solanum nigrum* suppressed the oxidant-mediated DNA sugar damage (Sultana et al., 1995), and the plant exerted cytoprotection against gentamicin-induced toxicity on Vero cells (Kumar et al., 2001)

and antineoplastic activity against Sarcoma 180 in mice (Yen et al, 2001). More recent studies revealed that extracts of *Solanum nigrum* induced apoptosis in MCF-7 cells (Son et al., 2003) and inhibited 12-O-tetradecanoylphorbol-13-acetate-induced tumor promotion in HCT-116 cells (Lee et al., 2004). The ethanol extract of dried fruits of *Solanum nigrum* had a remarkable hepatoprotective effect in CCl_4- induced liver damage (Raju et al., 2003) and water extract shows hepatoprotective effects against CCl_4-induced oxidative damage in rats (Lin et al., 2008). Aqueous extract of *Solanum nigrum* also inhibit growth of cervical carcinoma(U14) via modulating immune response of tumor bearing mice and inducing apoptosis of tumor cells (Li et al, 2008) while total alkaloids isolated from *Solanum nigrum* show antitumor effects *in vitro* and *in vivo* (Li et al, 2008a). Oral administration of *Solanum nigrum* extract significantly reduces thioacetamide-induced hepatic fibrosis in mice, probably through the

reduction of TGF-beta1 secretion (Hsieh et al, 2008). Cancer preventive lunasin from *Solanum nigrum* inhibits acetylation of core histone H3 and H4 and phosphorylation of retinoblastoma protein (Rb) (Jeong et al, 2007). Zakaria et.al. (2006) have confirmed the traditional claim of antinoceptive, anti-inflammatory and antipyretic effects of chloroform extract of *Solanum nigrum* in animal models.

Chemistry: Fruits of *Solanum nigrum* contain glucose, fructose and beta-carotene (15 to 20%). Seeds, comprising nearly 10% of the weight of the fresh fruits, contain 17.5% protein on a dry weight basis and 21.5% oil. The component fatty acids are 46% linoleic, 49.7% oleic, 1.8% palmitic, and 1.9% stearic (Duke, 1989).

The commonly occurring constituents of *Solanum nigrum* are tannins, saponins, alkaloids, ascorbic acid, beta-carotene, diosgenin, solanine, solasodine, etc. (Duke, 1989). The fruits are rich in alkaloids, glycoalkaloids, solanine, diosgenin, β-carotene, ascorbic acid, fructose and glucose (Duke, 1989). Wang et. al. (2007) have reported the occurrence of 6-methoxyhydroxy coumarin, syringaresinol-4-O-beta-D-glucopyranoside, pinoresinol-4-O-beta-D-glucopyranoside, 3, 4-dihydroxybenzoic acid, p-hydroxybenzoic acid and 3-methoxy-4-hydroxybenzoic acid. Steroidal

oligoglycosides (Ikeda et al, 2000) and two quercetin gycosides (Nawwar et al., 1989) were reported from *Solanum nigrum.*

Withania somnifera

Biological activity: ***Withania somnifera***, also known as **Ashwagandha, Indian ginseng, Winter cherry**, is a plant in *Solanaceae* nightshade family. *Withiania somnifera* is known as "Indian ginseng" (Kulkarni and Dhar, 2007), and has cardioprotective activity (Mohanty et al., 2008) and anti-inflammatory activity (Sumantran et al., 2008). In Ayurveda Ashwaganda is considered a rasayana herb, which works on a nonspecific basis to increase health and longevity. This herb is also considered an adaptogen which is a nontoxic herb that works on a nonspecific basis to normalize physiological function, working on the HPA axis and the neuroendocrine system. The roots and berries of the plant are used in herbal medicine. In Ayurveda, the fresh roots are sometimes boiled in milk, prior to drying, in order to leach out undesirable constituents. The berries are used as a substitute for rennet, to coagulate milk in cheese making.

Chemistry: Withanolides have been reported from *Withiania somnifera* (Ali et al., 1997, Misra et al., 2005, Dhar et al, 2006, Misra et al., 2008).

6.2. MATERIALS AND METHODS

6.2.1. Plant material and preparation of the extracts

The plant material of eight wild herbs were collected from Aligarh, U.P., India and identified by Dr.Athar Ali Khan, Department of Botany, Aligarh Muslim University, Aligarh, U.P., India.

The dried plant materials (10 g each) were extracted in alcohol, separately .The extracts were dried under reduced pressure using Buchi rotavapor.

6.2.2. Determination of total phenolic content

Total phenolic content was determined by Folin-Ciocalteau Method. The detailed procedure is described in Chapter 2.

6.2.3. Antioxidant activity measurement

Antioxidant activity was measured by determining the reducing power, DPPH radical scavenging, inhibitory effect on Fenton reaction induced oxidative DNA damage and inhibition of lipid peroxidation by the extracts of medicinal herbs. The detailed procedure is given in Chapter 2.

6.3. RESULTS

6.3.1. Yields of the extract

As shown in Table 6.1, the percent yield of the extracts range from 7.26% to 14.53%. There is no relation between the yield and antioxidant activity.

Table 6.1: Yield and total phenolic content of the ethanol extracts of medicinal herbs*.

Family	Herbs Studied	Part Used	Yield (%)	Total phenolic content (μg of GAE/μg of dry weight)
Asteraceae	*Pluchea lanceolata*	Aerial parts	9.87	82.05 ± 0.09
	Vernonia cinerea	Aerial parts including flowers	14.53	64.13 ± 0.14
Euphorbiaceae	*Acalypha indica*	Leaves	9.02	49.21 ± 0.07
	Phyllanthus fraternus	Leaves	12.39	76.08 ± 0.11
Scrophuliaraceae	*Lindenbergia indica*	Leaves	7.31	32.64 ± 0.17
	Scoparia dulcis	Aerial parts including flowers	7.26	45.13 ± 0.04
Solanaceae	*Solanum nigrum*	Edible fruits	11.41	106.48 ± 0.09
	Withiania somnifera	Fruits	8.28	59.27 ± 0.12

* Values are expressed as Mean ± S.E.M., n=4.

6.3.2. Total phenolic content

The total phenolic content of the medicinal herbs ranged from 32.64 to 106.43 µg of GAE/µg of dry weight (Table 6.1).

6.3.3. Reducing power

Figure 6.1 shows the reducing power of the ethanol extracts of medicinal herbs.The ethanol extracts of *Solanum nigrum* (SN) berries have got highest reducing power.

6.3.4. DPPH radical scavenging activity

As shown in Figure 6.2, ethanol extract of the berries of *Solanum nigrum* showed highest DPPH radical scavenging activity, followed by the leaf extracts of *Pluchea lanceolata* and *Phyllanthus fraternus*.

6.3.5. Superoxide radical scavenging assay

All the medicinal herbs possess scavenging effects on superoxide anion radical, in a concentration dependent manner (Figure 6.3). The fruit extract of *Solanum nigrum* is showing highest superoxide radical scavenging followed by the leaf extracts of *Pluchea lanceolata* and *Phyllanthus fraternus*. The leaf extract of *Lindenbergia indica* is showing lowest superoxide radical scavenging.

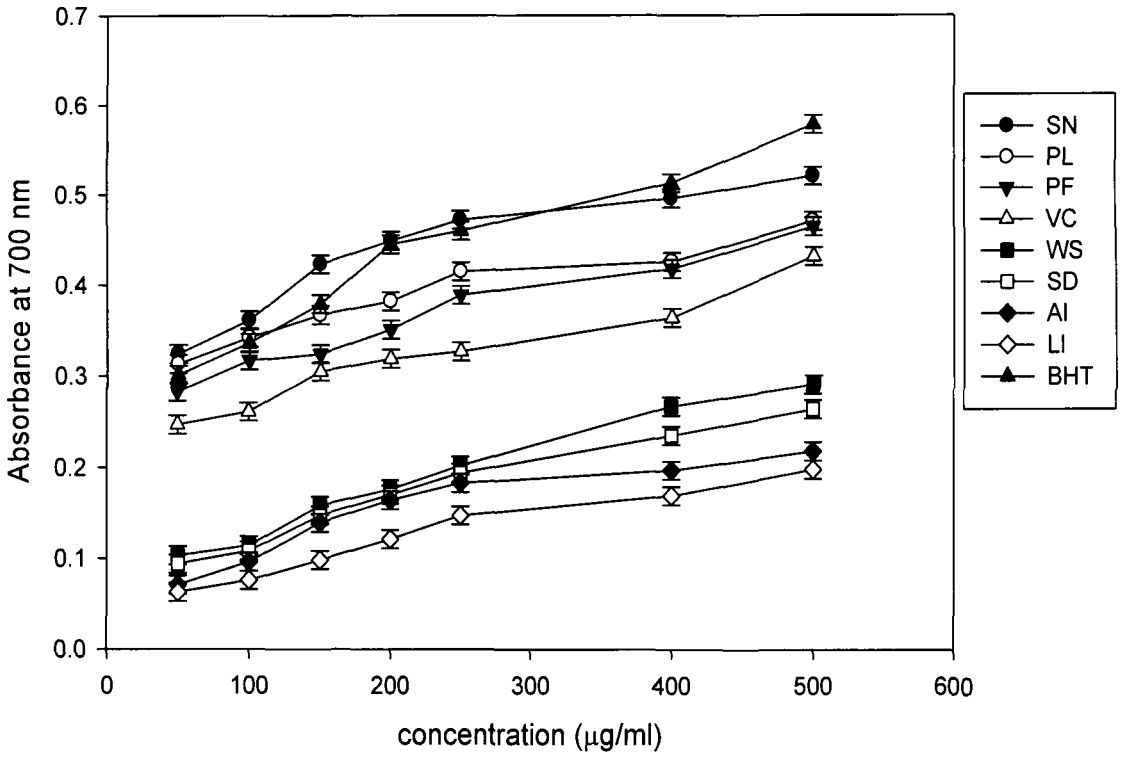

Figure 6.1: Reducing power of the ethanol extracts of medicinal herbs. BHT is used as a standard.Values are expressed as Mean ± S.E.M., n=3. SN=*Solanum nigrum*; PL=*Pluchea lanceolata*; VC=*Vernonia cinerea*; PF=*Phyllanthus fraternus*; AI=*Acalypha indica*; SD=*Scoparia dulcis*; LI=*Lindenbergia indica;* WS=*Withiania somnifera*.

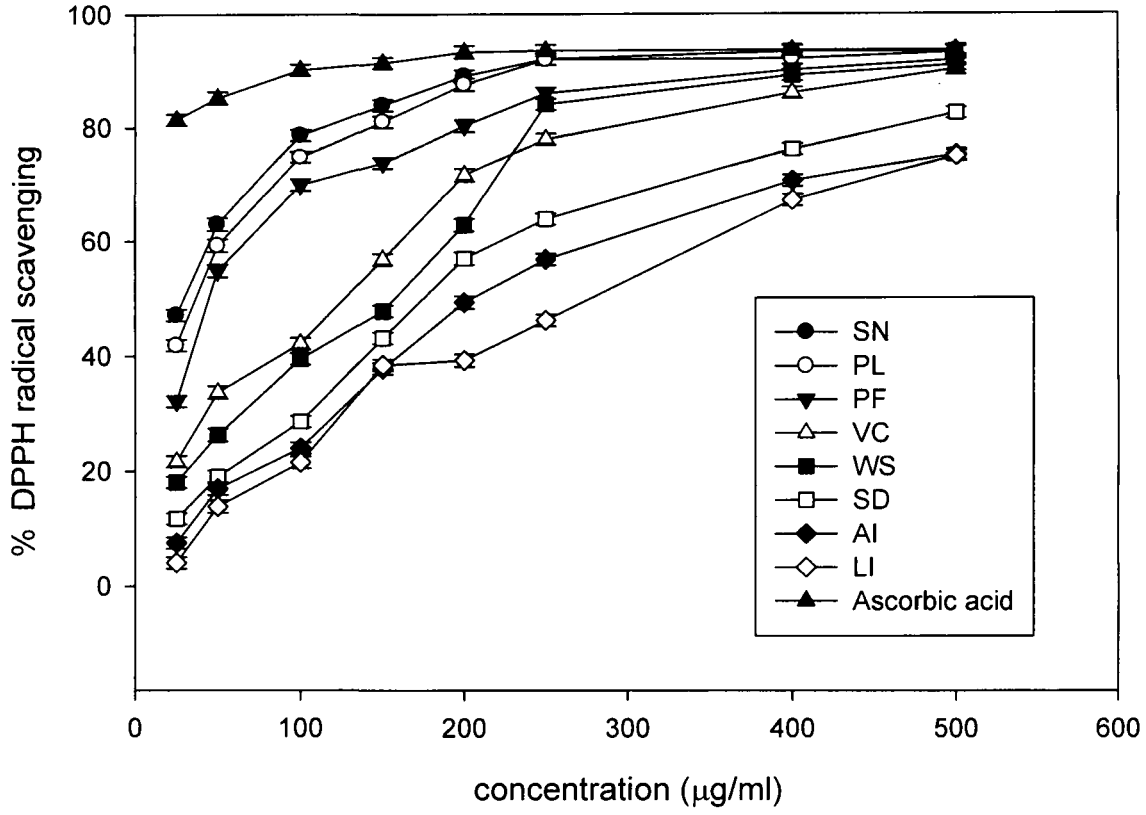

Figure 6.2: DPPH radical scavenging activity of the ethanol extracts of medicinal herbs. Ascorbic acid is used as a standard. Values are presented as Mean ± S.E.M., n=3. SN=*Solanum nigrum*; PL=*Pluchea lanceolata*; PF=*Phyllanthus fraternus*; VC=*Vernonia cinerea*;WS=*Withiania somnifera*; AI=*Acalypha indica*; SD=*Scoparia dulcis*; LI=*Lindenbergia indica.*

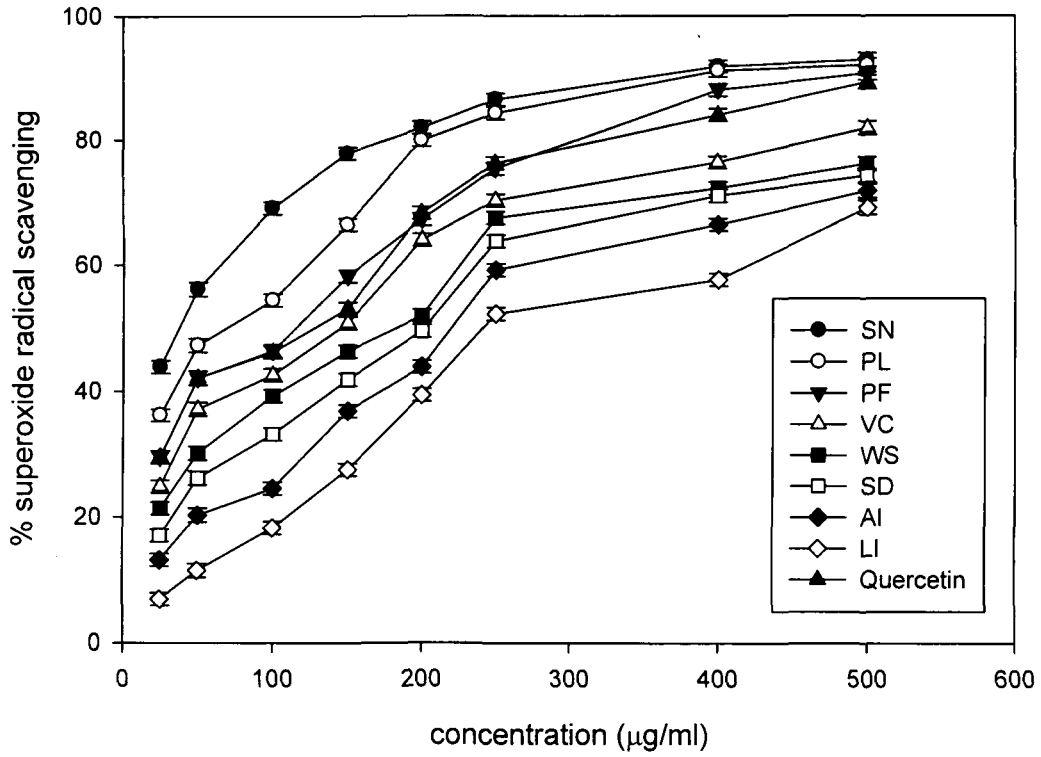

Figure 6.3: Superoxide radical scavenging activity of the ethanol extracts of medicinal herbs. Quercetin is used as a standard. Values are expressed as Mean ± S.E.M., n=3. SN=*Solanum nigrum*; PL=*Pluchea lanceolata*; PF=*Phyllanthus fraternus*; VC=*Vernonia cinerea*; WS=*Withania somnifera*; SD=*Scoparia dulcis*; AI=*Acalypha indica*; LI=*Lindenbergia indica.*

6.3.6. Inhibition of lipid peroxidation in egg yolk system

In this assay system also, *Solanum nigrum* showed highest antioxidant activity and inhibited lipid peroxidation by 76.92% at a concentration of 200 μg/ml (Figure 6.4).

6.3.7. Antioxidant Activity in β-carotene-linoleate system

As shown in Figure 6.5, all the medicinal herbs showed good antioxidant activity in β-carotene-linoleate system. The antioxidant activity is dependent on the concentration of the extracts taken. The antioxidant activity of the extracts of medicinal herbs in decreasing order is as follows: *Solanum nigrum* > *Pluchea lanceolata* > *Phyllanthus fraternus* > *Vernonia cinerea* > *Withania somnifera* > *Scoparia dulcis* > *Acalypha indica* > *Lindenbergia indica.*

6.4. DISCUSSION

Ethanol extracts of eight medicinal herbs of Aligarh, India, belonging to the family Asteraceae, Euphorbiaceae, Scrophuliaraceae and Solanaceae were evaluated for their antioxidant activity and total phenolic content.

Data presented in Table 6.1 indicates that the eight medicinal herbs varied considerably in their total phenolic content. The decreasing order of total phenolic content is as follows: *Solanum nigrum* > *Pluchea lanceolata* > *Phyllanthus fraternus* > *Vernonia cinerea* > *Withania somnifera* > *Scoparia*

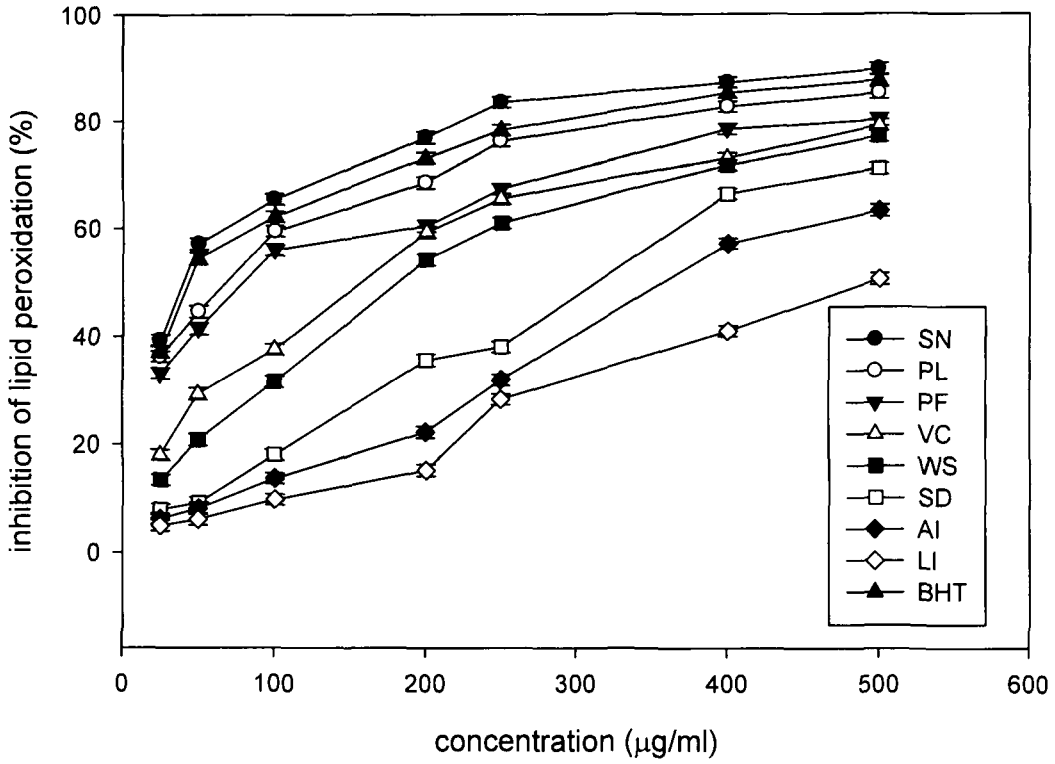

Figure 6.4: Inhibition of lipid peroxidation in egg yolk system induced by $FeSO_4$ by the ethanol extracts of medicinal herbs. BHT is used as a standard.Values are expressed as Mean ± S.E.M.,n=3.SN=*Solanum nigrum*; PL=*Pluchea lanceolata*;PF=*Phyllanthus fraternus*; VC= *Vernonia cinerea*; WS=*Withania somnifera;*SD=*Scoparia dulcis*; AI=*Acalypha indica*; LI=*Lindenbergia indica.*

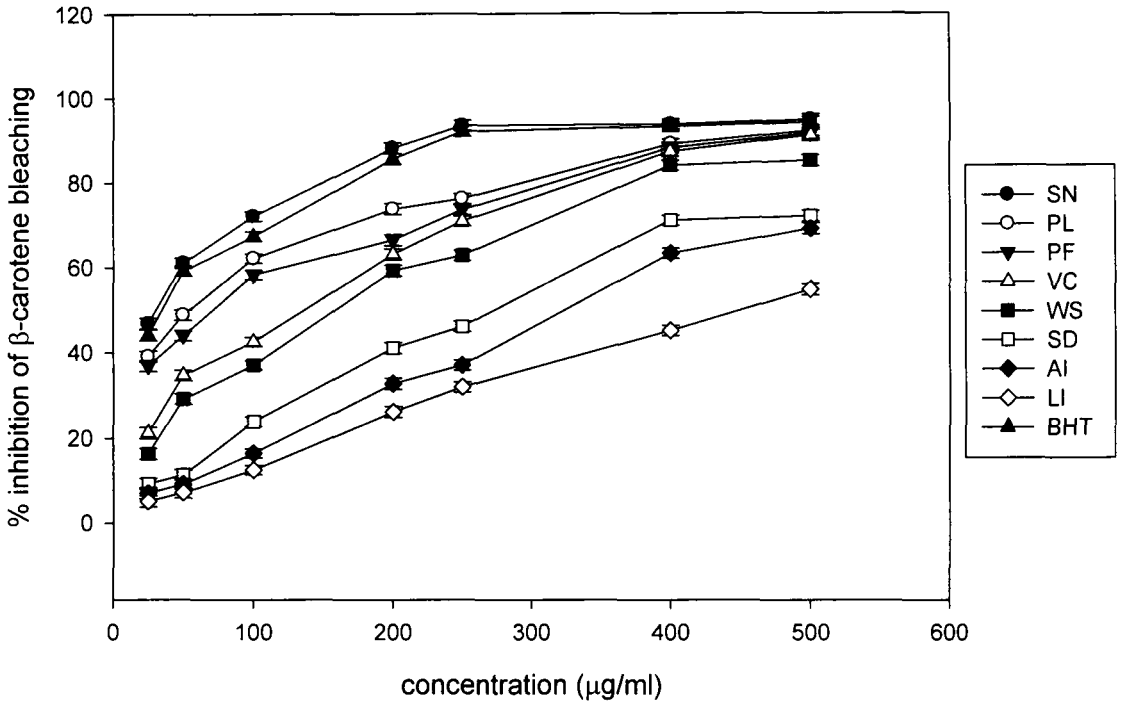

Figure 6.5: Antioxidant activity of the ethanol extracts of medicinal herbs as determined by β-carotene linoleate model. BHT is used as a standard. Values are expressed as Mean ± S.E.M., n=3. SN=*Solanum nigrum*; PL= *Pluchea lanceolata*; VC=*Vernonia cinerea*;WS=*Withania somnifera*; SD=*Scoparia dulcis*; AI=*Acalypha indica*; LI=*Lindenbergia indica.*

dulcis > *Acalypha indica* > *Lindenbergia indica*. Highest total phenol content was found in the berries of *Solanum nigrum* i.e., 106.48 µg of GAE/ µg of dry weight, while the lowest phenolic content was found in the leaves of *Lindenbergia indica* i.e. 32.64 µg of GAE/ µg of dry weight.

Figure 6.1, shows the reducing power of the ethanol extracts of medicinal herbs, *Solanum nigrum* is showing highest reducing power.

The results obtained from DPPH radical scavenging assay indicates that the ethanol extracts of medicinal herbs are reducing DPPH radical in a dose dependent manner (Figure 6.2). The DPPH radical scavenging activity was highest in the fruits of *Solanum nigrum*, followed by *Pluchea lanceolata*, *Phyllanthus fraternus*, *Vernonia cinerea*, *Withiania somnifera*, *Scoparia dulcis*, *Acalypha indica* and *Lindenbergia indica*. The same order is followed in superoxide radical scavenging activity (Figure 6.3), inhibition of lipid peroxidation in egg yolk system (Figure 6.4) and inhibition of lipid peroxidation in β-carotene-linoleate system (Figure 6.5).

A similarity has been observed in total phenolic content and antioxidant activity of some of the medicinal herbs belonging to different families while a difference is noticed within the family, for example, total phenolic content in the fruits of *Solanum nigrum* is 106.48 µg of GAE/µg of dry weight while

in the member of the same family *Withiania somnifera,* it is 59.27 μg of GAE/μg of dry weight.

A remarkable thing to notice is that the berries of *Solanum nigrum* are rich in alkaloids and saponins (Duke, 1989, Ikeda et al., 2000) rather than flavonoids (Nawwar et al., 1989), despite this they are showing higher antioxidant activity than the herbs rich in flavonoids, such as, *Scoparia dulcis* and *Lindenbergia indica.*

The highest antioxidant activity of the berries of *Solanum nigrum* could be attributable to the presence of β-carotene (Duke, 1989), phenolic acids and phenol glycosides (Wang et al. 2007) in addition to other radical scavenging compounds.

Second highest antioxidant activity is shown by the leaf extract of *Pluchea lanceolata.* The plant is used for the treatment of rheumatoid arthritis. The high antioxidant activity of this plant could be partially attributable to the presence of flavonoids (The Wealth of India, 1969). The phenolic compounds other than flavonoids may also be contributing towards the high antioxidant activity *Pluchea lanceolata.*

Phyllanthus fraternus, an herb belonging to the family Euphorbiaceae also showed high antioxidant activity and total phenolic content. At a concentration of 200μg/ml, the ethanol extract of *Phyllanthus fraternus*

scavenges 80.32% of DPPH free radical (Figure 6.2). It is a newly discovered species of Genus *Phyllanthus* and there is not much work done on the phytoconstituents of this plant. However, studies on its medicinal properties showed that it is quite efficient in the treatment of liver diseases (Kirtikar and Basu, 1975) and administration of the aqueous extract of *Phyllanthus fraternus* significantly decreased the thioacetamide (Padma and Setty, 1997) or carbon tetrachloride (Padma and Setty, 1999) induced lipid peroxidation *in vivo* and protected the liver from the hepatotoxin induced toxicity. It also possesses antifungal (Bhatnagar et al., 1961; Bhowmick and Chaudhari, 1982) anticancer (Dhar et al., 1968) and anti-diabetic (Ramakrishnan et al., 1982) activities.Thus, *Phyllanthus fraternus* is a medicinally important herb and some of its reported medicinal properties could be due to its high antioxidant activity.

Vernonia cinerea belonging to Family Asteraceae, also showed good antioxidant activity but at a higher concentration compared to *Solanum nigrum*, *Pluchea lanceolata* and *Phyllanthus fraternus*. For example, in DPPH radical scavenging assay, *Solanum nigrum*, *Pluchea lanceolata* and *Phyllanthus fraternus* scavenges 83.92%, 81.02% and 73.59% of DPPH free radical at a concentration of 150 µg/ml while *Vernonia cinerea* scavenges only 56.80 % of DPPH free radical at the concentration of 150 µg/ml. But

the radical scavenging activity of *Vernonia cinerea* increased significantly at higher concentration, it scavenges 71.46% of free radical at 200μg/ml.

The alcoholic extract of *Withiania somnifera* (Solanaceae) shows more than 50% DPPH radical scavenging activity (Figure 6.2) at 200 μg/ml and the radical scavenging activity suddenly rises to more than 80% at a concentration of 250 μg/ml. Fairly high superoxide radical scavenging activity (Figure 6.3), inhibition of the lipid peroxidation in egg yolk system (Fig. 6.4) and β-carotene model (Figure 6.5) is observed in alcoholic extract of *Withania somnifera* at a concentration of 200 μg/ml and above. *Withiania somnifera,* known as "Indian ginseng" (Kulkarni and Dhar, 2007), has cardioprotective activity (Mohanty et al., 2008) and anti-inflammatory activity (Sumantran et al., 2008). The withanolides, reported from *Withiania somnifera* (Misra et al., 2005, Misra et al., 2008) may be responsible for some of the reported biological activities, but the antioxidant phenolics (Table 6.1) could also be contributing to the medicinal properties of *Withiania somnifera,* either directly or by synergic effect.

Scoparia dulcis (Scrophuliaraceae), shows above 50% DPPH radical scavenging activity at a concentration of 200 μg/ml and shows fairly good activity in other assays (Figures 6.3, 6.4 and 6.5) which supports earlier reports (Babincova and Sourivong, 2001; Ratnasooriya et al., 2005). The

anti-inflammatory (Ahmad et al., 2001), antidiabetic (Latha and Pari, 2003, 2004, 2004a, 2004b, Pari and Latha, 2004, 2004a), antiulcer (Babincova et al., 2008), antiviral (Hayashi, K. et al., 1988), antimalarial (Riel et al., 2002) and anticancer (Ahsan et al., 2003) activity of *Scoparia dulcis* extracts may be attributable to novel terpenes (Hayashi, T. et al., 1990, 1990a,1992) and antioxidant flavonoids (Hayashi, T. et al., 1988; Li et al., 2004; Pereira-Martins et al., 1998).

Acalypha indica Linn. (Euphorbiaceae) shows above 50% DPPH radical scavenging activity at a concentration of 250 µg/ml and shows fairly good activity in other assays at only higher concentration of 400 µg/ml despite the presence of flavonoids (Nahrstedt et al., 2006).

Lindenbergia indica (L.) (Scrophulariaceae) shows lowest DPPH radical scavenging activity amongst all the herbs tested and same trend is observed in other assays although flavonoids are reported from this plant (Tiwari and Choudhary, 1980). Therefore, the presence of flavonoids is not the only criteria for determining potential source of natural antioxidants.

In conclusion all the medicinal herbs are showing good antioxidant activity especially *Solanum nigrum, Pluchea lanceolata* and *Phyllanthus fraternus*. The antioxidant activity of these medicinal herbs is attributable to several antioxidant compounds present in the extracts of these herbs such as

carotenoids, phenolic acids and flavonoids. Therefore, the herbal formulations based on the extracts of medicinal herbs can be used for the prevention and treatment of oxidative stress related disorders, such as, cancer, diabetes and cardiovascular diseases.

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CHAPTER –7

ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC CONTENT OF EDIBLE FLOWERS GROWN IN ALIGARH

7.1. INTRODUCTION

Diets rich in antioxidants are associated with lower risk of degenerative diseases such as coronary heart disease and cancer (Block et al., 1992). The reported carcinogenicity of synthetic antioxidants (Branen, 1975) has prompted a search for new sources of natural antioxidants, especially from fruits and vegetables forming part of human diet. Flowers are also used in diet in the form of salads, garnishes, drinks and jams, but there have not been any substantial study on their antioxidant activity. In the present study, the antioxidant activity of eleven commonly consumed edible flowers of Aligarh District was evaluated.

Flowers have been traditionally used in many types of cooking: European, Asian, Middle Eastern. Early American settlers also used flowers as food (Kowalchik, 1987; Belsinger, 1991). Today, there is a renewed interest in edible flowers for their taste, color, and fragrance. Edible flowers can be used fresh as a garnish or as an integral part of a dish, such as a salad. Some flowers can be stuffed or used in stir-fry dishes (Barash, 1993; Peterson, 1977). Edible flowers can be candied; frozen in ice cubes and added to beverages; made into jellies and jams; used to make teas or wines; minced and added to cheese spreads, herbal butters, pancakes, crepes, and

waffles (Shaudys, 1990). Many flowers can be used to make vinegars for cooking, marinades, or dressings for salad (Barash, 1993; Barash, 1997).

7.2. MATERIALS AND METHODS

7.2.1. Edible flowers

Edible flowers were grown for edible purpose, handpicked, dried at room temperature and made into powder form using kitchen blender. The powder is then brought into laboratory for preparing ethanol extracts.

7.2.2. Preparation of the flower extracts

The powdered petals (10 gm) from various edible flowers were extracted with 100 ml ethanol overnight in a shaker at room temperature. The extract was filtered through cheese cloth and the residue was re-extracted under the same conditions. The combined filtrate was evaporated in a rotary evaporation (Buchi rotavapor) below 40°C.

Table 7.1: List of edible flowers chosen for the study:

Flower's name	Type	Flower color	Bloom	Uses
Allium tuberosum	A	White	November to March	Garnish
Brassica campestris	A	Yellow	December to March	Garnish, salads
Coriandrum sativum	A	White	December to March	Garnish
Dianthus barbaratus	B	Purple	January to March	Garnish,Salads,jelly and jams
Dianthus caryophyllus	P	Pink	February & March	Garnish,salads,drinks ,jelly and jams
Hibiscus rosasinensis	P	Red, Pink	November to April	Salads, garnish, drinks, colouring
Petunia hybrida	P	White, Purple	February to April	Garnish, salad
Raphanus sativus	A	White, Pink	January to March	Salads, garnish
Rosa indica	P	Pink	November to August	Preparation of candy, sweet dishes and drinks, decoration on cakes.
Tropaeolum majus	P	Orange-red	January to March	Garnish, salads, as a substitute of capers
Viola wittrockiana	A	Violet, pink, white, yellow	February & March	Decorations on cakes

A = Annual; B = Biennial; P = Perennial

7.2.3. Determination of total phenolic content

Total phenolic content was determined by Folin-Ciocalteau Method. It is described in detail in Chapter 2.

7.2.4. Antioxidant activity measurement

Antioxidant activity was measured by determining the reducing power, DPPH radical scavenging, superoxide scavenging assay, inhibitory effect on Fenton reaction induced oxidative damage and inhibition of lipid peroxidation. The procedures are described in detail in Chapter 2.

7.3. RESULTS

7.3.1. Yield of the flower extracts

The percentage (%) yield of the flower extracts is shown in Table 7.2, ranges from 7.21 % to 4.28%.

7.3.2. Total phenolic content

As shown in Table 7.2, all the extracts of edible flowers have got good amount of total phenolic content ranging from 124.06 μg to 98.06 μg of GAE/μg of dry weight. Among these edible flowers *Tropaeolum majus* is

showing highest total phenolic content while the flower extract of *Brassica campestris* is having lowest total phenolic content.

Table 7.2: Percentage yield and total phenolic content of edible flowers.

Edible flowers	Yield (%)	Total phenolic content (μg of GAE/μg of dry weight)
Tropaeolum majus (TM)	7.21	124.06 ± 0.09
Viola wittrokiana (VW)	6.53	105.32 ± 0.04
Rosa indica (RI)	7.19	97.23 ± 0.08
Dianthus caryophyllus (DC)	4.28	93.04 ± 0.12
Hibiscus rosasinensis (HR)	8.61	91.52 ± 0.13
Dianthus barbatus (DB)	6.72	89.17 ± 0.05
Petunia hybrida (PH)	4.33	86.18 ± 0.07
Raphanus sativus (RS)	5.84	84.69 ± 0.12
Coriandrum sativum (CS)	6.09	80.12 ± 0.11
Brassica campestris (BC)	5.47	74.08 ± 0.05
Allium tuberosum (AT)	4.91	98.06 ± 0.21

7.3.3. Reducing power

As shown in Figure 7.1, the flower extract of *Tropaeolum majus* is possessing highest reducing power. The decreasing order of the reducing power of edible flower extracts is as follows: *Tropaeolum majus* > *Viola*

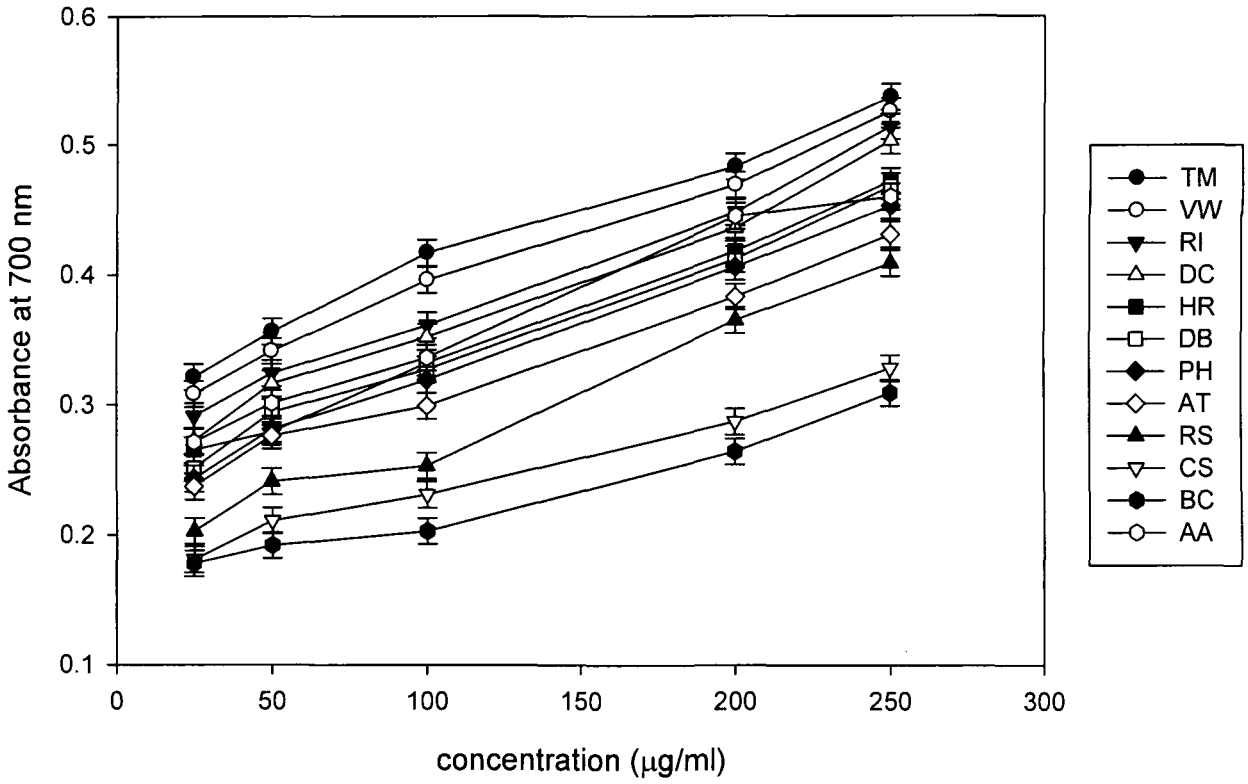

Figure 7.1: Reducing power of the ethanol extracts of edible flowers. Values are expressed as Mean ± S.E.M., n=3.TM=*Tropaeolum majus*; VW=*Viola wittrokiana*; RI=*Rosa indica*; DC=*Dianthus caryophyllus*; HR=*Hibiscus rosasinensis*;DB=*Dianthus barbatus*;PH=*Petunia hybrida*; AT=*Allium tuberosum*; RS=*Raphanus sativus*; CS=*Coriandrum sativum*; BC=*Brassica campestris*; AA= L-Ascorbic acid.

wittrokiana > *Rosa indica* > *Dianthus caryophyllus* > *Hibiscus rosasinensis* > *Dianthus barbatus* > *Petunia hybrida* > *Allium tuberosum* > *Raphanus sativus* > *Coriandrum sativum* > *Brassica campestris.*

7.3.4. DPPH radical scavenging activity

Figure 7.2 show that *Tropaeolum majus* has got highest radical scavenging activity. Lowest activity is shown by the flower extracts of *Brassica campestris*. The free radical scavenging activity of the extracts in descending order is as follows: *Tropaeolum majus* > *Viola wittrokiana* > *Rosa indica* > *Dianthus caryophyllus* > *Hibiscus rosasinensis* > *Dianthus barbatus* > *Petunia hybrida* > *Allium tuberosum* > *Raphanus sativus* > *Coriandrum sativum* > *Brassica campestris*.

7.3.5. Superoxide radical scavenging activity

In this assay, flower extract of *Tropaeolum majus*, commonly known as Nasturtium showed highest superoxide radical scavenging activity followed by *Rosa indica, Viola wittrokiana, Hibiscus rosasinensis, Dianthus caryophyllus, Allium tuberosum*, *Dianthus barbatus, Raphanus sativus, Coriandrum sativum* and *Brassica campestris* (Figure 7.3).

7.3.6. Antioxidant Activity in β-carotene-linoleate system

As shown in Figure 7.4, the flower extract of *Tropaeolum majus* (TM) is showing highest antioxidant activity. At a concentration of 100 μg/ml, it

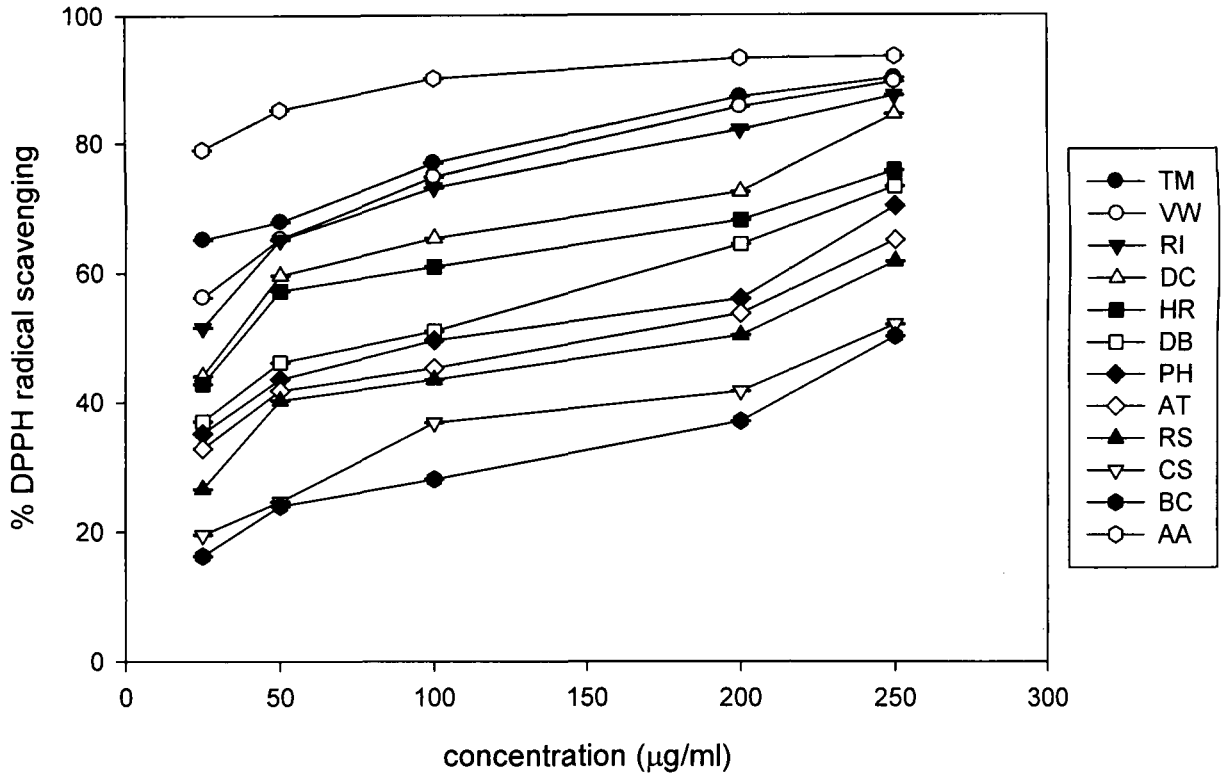

Figure 7.2: DPPH radical scavenging activity of ethanol extracts of edible flowers.Ascorbic acid is used as a standard. Values are expressed as Mean ± S.E.M., n=3. TM=*Tropaeolum majus*; VW=*Viola wittrokiana*; RI=*Rosa indica*; DC=*Dianthus caryophyllus*; HR=*Hibiscus rosasinensis*;DB=*Dianthus barbatus*; PH=*Petunia hybrida*; AT=*Allium tuberosum*; RS=*Raphanus sativus*; CS=*Coriandrum sativum*;BC=*Brassica campestris*; AA= L-Ascorbic acid.

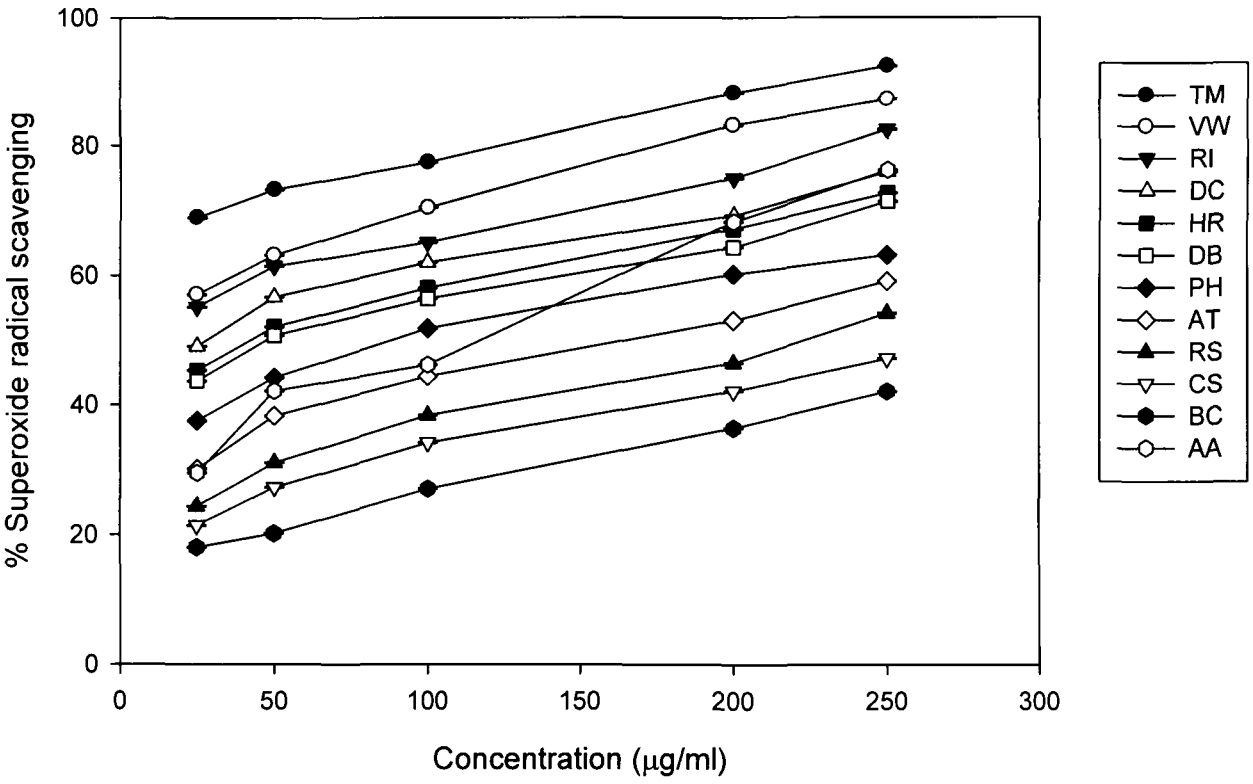

Figure 7.3: Superoxide radical scavenging activity of ethanol extracts of edible flowers. Values are expressed as mean ± S.E.M., n=3.TM= *Tropaeolum majus*; VW=*Viola wittrokiana*; RI=*Rosa indica*; DC=*Dianthus caryophyllus*; HR=*Hibiscus rosasinensis*;DB=*Dianthus barbatus*; PH=*Petunia hybrida*; AT=*Allium tuberosum*; RS=*Raphanus sativus*; CS=*Coriandrum sativum*; BC=*Brassica campestris.*

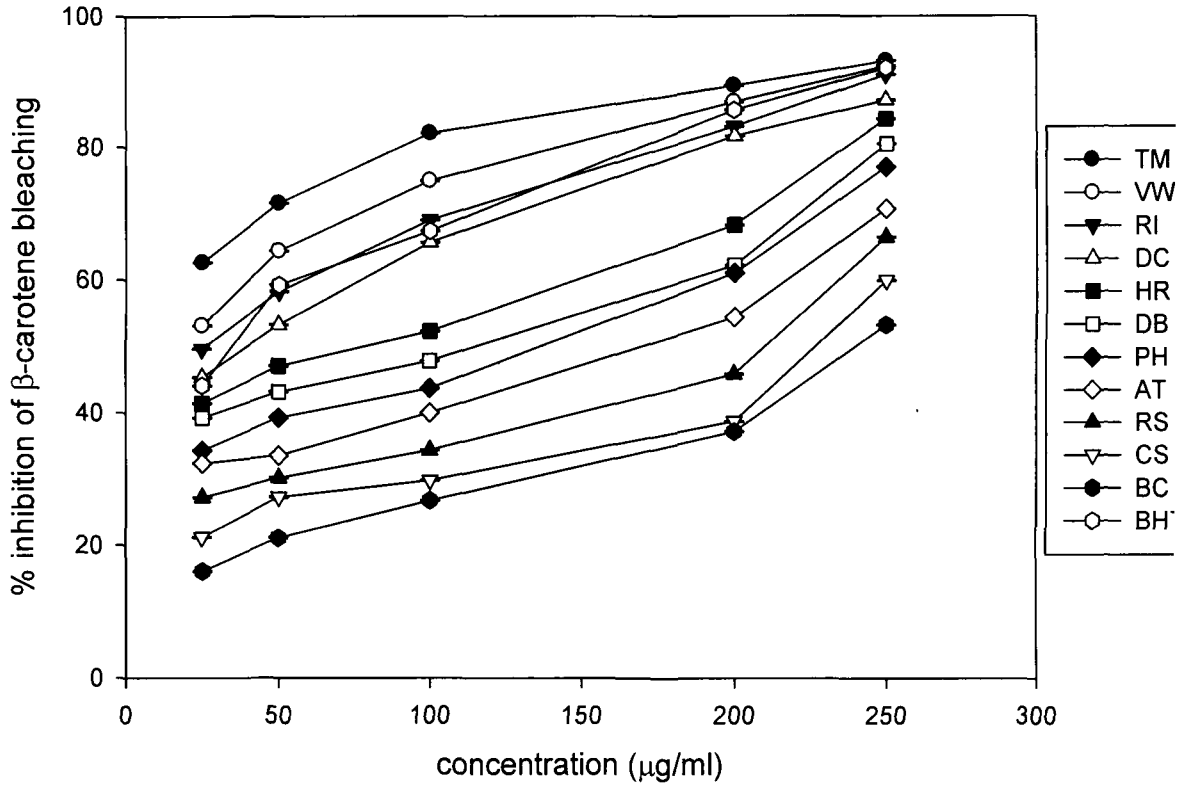

Figure 7.4: Antioxidant activity of the ethanol extracts of edible flowers asdetermined by β–carotene linoleate model. Values are expressed as Mean ± S.E.M., n=3. TM= *Tropaeolum majus*; VW=*Viola wittrokiana*; RI=*Rosa indica*; DC=*Dianthus caryophyllus*; HR=*Hibiscus rosasinensis*; DB=*Dianthus barbatus*; PH=*Petunia hybrida*;AT=*Allium tuberosum*; RS=*Raphanus sativus*; CS=*Coriander sativum*;BC=*Brassica campestris.*

inhibited the bleaching of β-carotene by 82.05 %.The flower extract of *Viola wittrokiana* (VW) inhibited 74.86% of β-carotene bleaching at a concentration of 100 µg/ml. The extracts of *Rosa indica*, *Dianthus caryophyllus*, *Hibiscus rosasinensis*, *Dianthus barbatus*, *Petunia hybrida*, *Allium tuberosum*, *Raphanus sativus, Coriandrum sativum* and *Brassica campestris* showed 68.95%, 65.61%, 52.24%, 47.81%, 43.67%, 39.93%, 34.28%, 29.74% and 26.69% inhibition of β-carotene bleaching, respectively, at 100 µg/ml.

7.4. DISCUSSION

As demonstrated by the results of this study (Table 7.2, Figures 7.1, 7.2, 7.3 and 7.4), edible flowers are having high antioxidant activity and total phenolic content, especially the flowers of *Tropaeolum majus,* which is one of the most widely used edible flowers and is a rich source of Lutein (Niizu and Rodriguez-Amaya, 2006), which helps in reducing the risk of cataract and macular degeneration. The high antioxidant activity of *Tropaeolum majus* is attributable to the presence of carotenoids, anthocyanins and phenolic compounds.

The flowers of Pansy (*Viola wittrokiana*) and Rose (*Rosa indica*) are also showing very high antioxidant activity (Figure 7.2, Figure 7.3 and Figure 7.4). Pansy (*Viola wittrokiana*) is a colorful addition to salads.

Besides this, it has got several medicinal properties, it is a expectorant that has been used to treat lung problems (bronchitis, dry cough and asthma), as well as numerous skin problems. Recently, its polar antioxidants were analyzed by HPLC method and flowers of Pansy (*Viola wittrokiana*) were reported to be promising source of natural antioxidants (Vukics et al., 2008). Rose is also a widely used edible flower and is used in various sweet preparations. Its petals are also used in the preparation of tea. Vinokur et al (2006) found that the tea prepared from the European rose cultivars are rich source of antioxidants. However, there is no such study done on Indian rose cultivar *Rosa indica*. In our study, *Rosa indica* is showing good antioxidant activity in all the assays.

Dianthus caryophyllus is ranking fourth in total phenolic content and antioxidant activity in the eleven edible flowers tested , it is used in the preparation of sweetened drinks. Phytochemical studies on the flowers of *Dianthus caryophyllus* showed the presence of malyl anthocyanins (Nakayama et al., 2000), 3, 5-Di-*O*-(β-glucopyranosyl) pelargonidin 6″-*O*-4, 6‴-*O*-l-cyclic malate and 3, *5*-di-*O*-(β-glucopyranosyl) cyanidin 6″ -*O*-4, 6‴-*O*-l-cyclic malate. These compounds may be largely responsible for good antioxidant activity.

Hibiscus rosasinensis exhibits good antioxidant activity and has quite high total phenolic content. *Hibiscus rosasinensis* Linn. (Family Malvaceae), is widely distributed throughout the world. The alcoholic extract of flowers of *H.rosasinensis* has been reported to possess anticonvulsant property (Kasture et al., 2000). Powdered leaves of *H.rosasinensis* showed lowering of blood pressure (Agarwal and Shinde, 1967).Ethanolic extract of *Hibiscus rosasinensis* petal inhibited carbon tetrachloride induced lipoperoxidation in rats (Obi and Uneh, 2003). It showed wound healing activity in a preclinical study on Sprague Dawley Rat (Nayak et al., 2007).Siddiqui et al. (2006) studied the hypotensive activity of the hydroalcoholic extracts of *Hibiscus rosasinensis* and found that the crude extracts exhibited prominent activity when compared to the standard minoxidil.

Among all the tested edible flowers, top eight edible flowers showing high antioxidant activity are *Tropaeolum majus, Viola wittrokiana*, *Rosa indica*, *Dianthus caryophyllus*, *Hibiscus rosasinensis*, *Dianthus barbatus*, *Petunia hybrida* and *Allium tuberosum*. Between *Dianthus caryophyllus* and *Dianthus barbatus*, *Dianthus caryophyllus* (DC) is showing higher antioxidant activity and total phenolic content. The pink coloured variety of carnation (*Dianthus caryophyllus*) which was used in the study showed good antioxidant activity in all the assays including DPPH and superoxide anion

radical scavenging assay (Figure 7.2 and 7.3) , *Dianthus caryophyllus* is native to Europe and India. Petals from the fresh flowers of *Dianthus caryophyllus* are decorative addition to salads, soups, and open sandwiches. An herbal vinegar from the flowers turn distilled vinegar a delicate pink and imparts it's decidedly clove like taste (Keville, 1991). The total phenolic content in the flowers of *Petunia hybrida* (Table 7.2) is 86.18 (µg of GAE/ µg of dry weight), it shows good antioxidant activity in all the assays (Figure 7.2, 7.3 and 7.4) which could be attributable to the presence of flavonoid compounds (Yamazaki et al., 2002;Zerback et al.,1989). Shvets et al. (1996) isolated steroidal glycosides from the seeds of *Petunia hybrida.*

Flowers of *Allium tuberosum* also showed good amount of total phenolics and antioxidant activity. *Allium tuberosum* is a medicinally useful plant; it contains thiosufinates which is reported to induce apoptosis in PC-3 human prostate cancer cells. (Kim et al., 2008). The taste of garlic blossoms is milder than garlic bulb and it makes excellent salad. The flower extracts of *Raphanus sativus*, *Coriandrum sativum* and *Brassica campestris* also showed considerable antioxidant activity. At a concentration of 250 µg/ml, DPPH radical scavenging activity of the flowers of *Raphanus sativus*, *Coriandrum sativum* and *Brassica campestris* is 61.72%, 52.06% and 50.21%, respectively.

In conclusion, all the edible flowers are showing good amount of antioxidant activity and high total phenolic content. Thus, edible flowers not only add colour to any dish, their use in food can also add to the nutritive value of the food as they are rich source of antioxidants.

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PUBLICATIONS

1. Antioxidant activity of water and alcohol extracts of *Thuja orientalis* leaves.2007. I. Nizam and M. Mushfiq. *Oriental Pharmacy and Experimental Medicine*, 7, 65-73.
2. Antioxidant Activity and Total Phenolic Content of Five Different Solvent Extracts of the Fruit of *Ficus racemosa* .2009. I. Nizam and M. Mushfiq. *Journal of Agricultural and Food Chemistry*. (Communicated).

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Antioxidant activity of water and alcohol extracts of *Thuja orientalis* leaves

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SUMMARY

Water and alcohol extracts were prepared from dried and powdered leaves of *Thuja orientalis (T. orientalis)*. The reducing power, total phenolic content, the 1,1-diphenyl-2-picrylhydrazyl scavenging activity, inhibitory effect on Fe (II)-EDTA-H_2O_2 (Fenton reaction system) induced DNA damage and inhibitory effect on human red blood cell (RBC) hemolysis were evaluated in the present study. At a concentration of 200 mg, water and alcohol extracts of *T. orientalis* inhibited the hydrolysis of DNA by 72.859% and 65.312%, respectively. Water and alcohol extracts of *T. orientalis* also inhibited 2,2'-Azobis(2-amidinopropane) dihydrochloride induced RBC hemolysis to the extent of 69.30% and 54.55%, respectively. The reducing power and antioxidative activity of water extract was found to be more than that of alcohol extract. This is attributable to the presence of higher amount of phenolic compounds in water extract. The present results indicate that the *T. orientalis* extracts are rich sources of natural antioxidants and can protect DNA and human red blood cells against free radical induced oxidative damage.

Key words: *Thuja orientalis*; Reducing power; 1,1-diphenyl-2-picrylhydrazyl; Scavenging activity; Antioxidative activity; DNA damage; RBC hemolysis

INTRODUCTION

The reactive oxygen species (ROS), superoxide ion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$) are involved in various human diseases, such as, Alzheimer's disease, aging, cancer, inflammation, rheumatoid arthritis and atherosclerosis (Freeman, 1984; Singh, 1989; Squadrito and Pryor, 1998; Pryor, 2000). Several studies have demonstrated a correlation between the antioxidant properties of phytoconstituents with oxidative stress defense (Aruoma, 2003; Amarowicz *et al.*, 2004). Recently, there is an impetus for search of powerful and non-toxic antioxidants from natural sources, especially crude drugs derived from medicinal plants (Ramarathnam *et al.*, 1997). The plant phenolics have the ability to scavenge free radicals by single-electron transfer (Hirano *et al.*, 2001). Such natural antioxidants could prevent the formation of ROS-related disorders in human beings, making them safer than synthetic antioxidants, which may be carcinogenic and harmful to the lungs and liver (Branen, 1975).

Thuja orientalis L. [syn. *Platycladis stricta* Spach; *Platycladis orientalis* (L.) Franco; *Biota orientalis* (L.) Endl.], commonly known as Oriental Thuja, Oriental Arbor Vitae and Chinese Arbor Vitae, is an ornamental conifer of the cypress family. The name 'Arbor vitae' is from Latin, 'tree of life', and is related to long life and vitality in Buddhist thoughts in China. The leaves of *Thuja orientalis* L [Syn. *Biota orientalis* (L.) Endl. (Cupressaceae)] have been used in Chinese medicine for treatment of gout, rheumatism,

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cuvette, and the solutions were mixed again. After 30 s and before 8 min, 300 μl of a 20% sodium carbonate solution was added. The solutions were left at room temperature for 2 h. Then the absorbance of the developed blue color was determined at 765 nm. The amount of light absorbed is proportional to the amount of oxidizable material present, that is, phenolic compounds. Gallic acid was used as a standard for the calibration curve. The total phenolic content is reported (Table 1) as gallic acid equivalents (mg) using the following linear equation based on the calibration curve:

$A = 0.0011x + 0.0025 \quad R^2 = 0.9995$

Where A is the absorbance and x is the gallic acid equivalents (μg).

Reducing power

Total reducing power was determined as described by Zhu *et al.* (2002). *Thuja* extracts (50 - 500 μg) in 1 ml of distilled water were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$]; the mixture was then incubated at 50°C for 30 min. Afterward, 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3,000 rpm for 10 min. Finally, 2.5 ml of the upper layer solution was mixed with 2.5 ml of distilled water and 0.5 ml $FeCl_3$ (0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

DPPH radical scavenging activity

DPPH radical scavenging activity was evaluated by the method of Nagai *et al.* (2003). The assay mixture contained 0.3 ml of 1.0 mM DPPH radical solution, 2.4 ml of 99% ethanol, and 0.3 ml of extract solution of concentrations varying from 50 mg to 500 mg. The solutions were rapidly mixed and scavenging capacity was measured spectrophotometrically by monitoring the decrease in absorbance at 517 nm. Ascorbic acid (1 mM) was used as positive control while reaction mixture (DPPH radical solution) minus extract solution was taken as control. The percent (%) radical scavenging was calculated by the following equation.

Where A_c = Absorbance of control at 517 nm and A_s = Absorbance of sample at 517 nm.

Inhibition of Fe (II)-EDTA-H_2O_2 induced oxidative DNA damage

Solution of DNA was prepared by dissolving 2 mg of calf thymus DNA (Sigma Chemical Company, St. Loius, MO) in 1 ml of 10 mM tris-HCl pH 7.4, 500 μg DNA and varying concentration of extract (25 - 250 μg) solution, 0.08 mM EDTA, 0.08 mM $FeSO_4$, 0.03% H_2O_2 and 20 mM Na-ascorbate. All solutions were sterilized before use. After incubation at 37°C for 1 h S_1 nuclease digestion was performed as described by Rahman *et al.* (1989). The assay determines the acid soluble nucleotides released from DNA because of enzymatic digestion. Acid soluble nucleotides were determined colorimetrically using the diphenylamine method of Schneider (1957). To a 1.0 ml aliquot, 2.0 ml of diphenylamine reagent (freshly prepared by dissolving 1 g of recrystallized diphenylamine in 100 ml of glacial acetic acid and 2.75 ml of conc. H_2SO_4) was added. The tubes were heated in a boiling water bath for 20 min. The intensity of blue color was read at 600 nm.

RBC hemolysis

Blood was obtained from healthy human donor and collected into heparinized tubes through the Blood Bank, J. N. Medical College, Aligarh Muslim University, Aligarh. Erythrocytes were separated from plasma and the buffy coat, and washed three times with 5 volumes of phosphate buffered saline (PBS), pH 7.4. During every wash, erythrocytes were centrifuged at 4,000 rpm for 10 min to obtain a packed cell preparation (Miki *et al.*, 1987). After the last wash, the packed RBC was suspended in four volumes of PBS solution. RBC oxidative

radical. Water extract of *Thuja orientalis* at a concentration of 500 mg was able to scavenge 68.59% of DPPH free radical while alcohol extract at the similar concentration was able to scavenge 59.73% of DPPH free radical. Thus, water extract is a better scavenger of DPPH free radical as compared to alcohol extract.

Inhibition of Fe (II)-EDTA-H_2O_2 induced oxidative DNA damage

We studied alcohol and water extracts of *Thuja orientalis* for their ability to modulate DNA damage produced by Fenton reaction. Control experiments (not shown) established that heat denatured DNA underwent 100% hydrolysis following treatment with S_1 nuclease, whereas native DNA resulted in < 10 % conversion. S_1 nuclease hydrolysis of DNA decreases in a dose dependent manner with the increasing concentration of water and alcohol extracts of *Thuja orientalis* (Fig. 3). At a concentration of 250 μg, water and alcohol extracts of *Thuja orientalis* inhibited the hydrolysis of DNA by 72.859% and 65.312%, respectively (Fig. 4). It shows that water extract is a stronger inhibitor of DNA cleavage, because it inhibits DNA degradation to a greater extent than the alcohol extract at all concentrations tested. Thus, *Thuja orientalis* extracts were able to prevent DNA hydrolysis and oxidative damage to DNA.

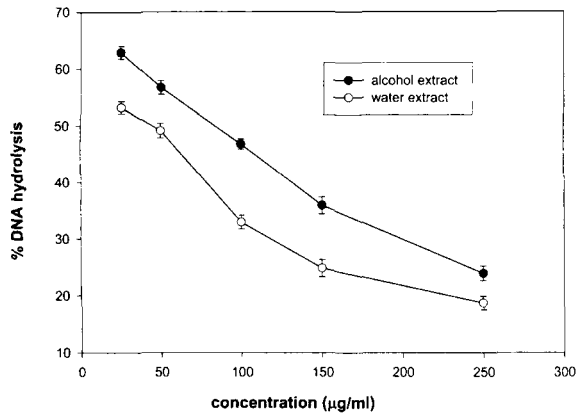

Fig. 3. Decrease in degradation of DNA in the presence of water and alcohol extracts of *Thuja orientalis*. All the points represent mean of triplicate samples and bars representing standard error of mean are shown.

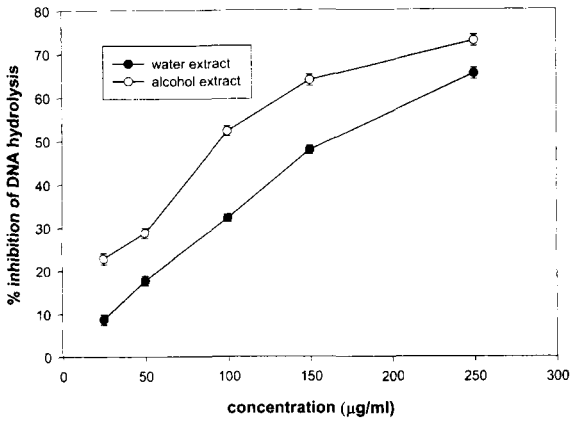

Fig. 4. Inhibition of DNA hydrolysis by water and alcohol extracts of *Thuja orientalis*. All the points represent mean of triplicate samples and bars representing standard error of mean are shown.

RBC hemolysis

Lipid oxidation of RBC membrane mediated by AAPH induces membrane damage and subsequent hemolysis (Miki *et al.*, 1987). Both water and alcohol extracts of *Thuja orientalis* showed inhibition of RBC hemolysis. As shown in Table 2, at a concentration of 12.5 μg/ml, water and alcohol extracts inhibited RBC hemolysis to the extent of

Table 2. Inhibitory effect (%) of water and alcohol extracts of *Thuja orientalis* on AAPH induced hemolysis of human RBC

Compound/extract	12.5 mg/ml	25 mg/ml	50 mg/ml
Ascorbic acid	80.44 ± 0.42	87.76 ± 0.75	92.38 ± 0.80
Water extract	69.30 ± 0.25	75.15 ± 0.84	88.14 ± 1.09
Alcohol extract	54.55 ± 0.66	68.34 ± 0.92	79.21 ± 1.31

Data are expressed as mean ± standard error of mean, n = 5.

cell types. Excessive oxidative damage to cellular membranes contributes to the initiation and progression of numerous degenerative diseases, including certain types of cancer and cardiovascular diseases (Pryor, 2000; Young and Woodside, 2001). Red blood cells are vulnerable to lipid peroxidation due to their high content of polyunsaturated lipids, their rich oxygen supply, and the presence of transition metals. Lipid oxidation of human red blood cell membrane mediated by AAPH induces membrane damage and subsequent hemolysis (Miki *et al.*, 1987; Zhu *et al.*, 2002). *Thuja orientalis* extracts inhibited AAPH (an azo peroxyl radical initiator) mediated human red blood cell hemolysis. At a concentration of 12.5 μg/ml, water and alcohol extracts inhibited RBC hemolysis to the extent of 61.30% and 54.55%, respectively. At a concentration of 50 μg/ml, water extract inhibited RBC hemolysis by 88.14%, which is only 4% less than L-ascorbic acid at similar concentration. Alcohol extract at the similar concentration showed inhibition by 79.21%, which is 9% less than water extract. This may again be attributable to higher amount of polyphenolic compounds in water extract. Thus, there is direct relation between phenolic content and antioxidant activity. Since *Thuja orientalis* extracts showed protection against lipid peroxidation of RBC membrane; it can also be used in the prevention of cardiovascular diseases.

The data reported in the present study demonstrates that water and alcohol extracts of *Thuja orientalis* have free radical scavenging activity and reducing power, and can provide protection against DNA oxidation and RBC hemolysis. Therefore, the herbal formulations based on *Thuja orientalis* extracts can be used for the prevention and treatment of oxidative stress related disorders, such as, cancer, vascular diseases, gout and rheumatism.

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